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CHEMICAL CONSTITUTION AND ANTI-TUBERCULOUS ACTIVITY:

PART IV. THIOSEMICARBAZONES AND RELATED COMPOUNDS.

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Received for publication September 19, 1955

THE anti-tuberculous activity *in vitro* of thiosemicarbazones was reported first by Domagk, Behnisch, Mietzsch and Schmidt (1946) and Behnisch, Mietzsch and Schmidt (1948), and subsequently by Hoggarth, Martin, Storey and Young (1949), Donovick, Pansy, Stryker and Bernstein (1950) and Hamre, Bernstein and Donovick (1950). The large number of thiosemicarbazones of substituted benzaldehydes examined did not include any derivatives containing the diphenyl structure. In view of the sixteen-fold increase in anti-tuberculous activity on passing from aniline to 4-aminodiphenyl (Cymerman-Craig, Rubbo and Pierson, 1954), it was of interest to test diphenyl-4-aldehyde thiosemicarbazone (I; R = H), and some related substances. To test the specificity of the intact thiosemicarbazone group (-CH : N — NH·CS·NH_2), some related thioureas (aryl-NH·CS·NH_2) were also examined, being the simplest derivatives possessing the thioureide group attached to an aromatic ring.

The results of the investigation of twenty-four compounds belonging to these two series are described in this paper.

MATERIALS AND METHODS.

Organic chemistry.

A number of the compounds listed in Tables I and II were previously unknown and were synthesised for this work by Cymerman-Craig and Loder (unpublished work) and Cymerman-Craig and Willis (1955). All test substances were of analytical purity.

Bacteriological.

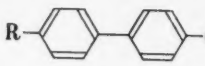
The tuberculostatic tests were performed by the serial dilution technique using *Mycobacterium tuberculosis* H37Rv as described by Cymerman-Craig *et al.* (1954). Bacteriostatic tests with *Staphylococcus aureus*, *Bacterium coli* and *Pseudomonas pyocyanea* were done as described by Rubbo, Albert and Maxwell (1942).

RESULTS.

In vitro *Activities.*

Thiosemicarbazones.

Table I gives the results of the examination of 13 thiosemicarbazones of aromatic carbonyl compounds. The thiosemicarbazones of 4-ethylsulphonyl- and



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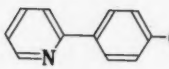

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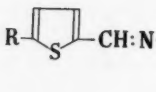

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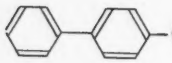

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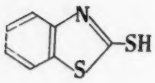

(V)

TABLE I.—*Thiosemicarbazones.*

Highest dilution expressed as 1/molarity of the substance completely inhibiting growth.

Lab. ref. number.	Thiosemicarbazone of—	R : N-NHCSNH$_2$ R =.	Organisms and media. H37Rv serum synthetic medium.	Organisms and media. H37Rv synthetic medium.
102	4-Acetamidobenzaldehyde	$CH_3CONH.C_6H_4.CH$:	8,000[1]	8,000[1]
143	4-Ethylsulphonylbenzaldehyde	$EtSO_2.C_6H_4.CH$:	16,000[2]	16,000[2]
130	Diphenyl-4-aldehyde	$Ph.C_6H_4.CH$:	512,000	512,000
169	4′-Methoxydiphenyl-4-aldehyde	$MeO.C_6H_4.C_6H_4.CH$:	512,000	512,000
164	4-Acetyl-4′-methoxydiphenyl	$MeO.C_6H_4.C_6H_4.CMe$:	128,000	256,000
165	4-Acetyl-4′-hydroxydiphenyl	$HO.C_6H_4.C_6H_4.CMe$:	256,000	512,000
155	4′-Carboethoxydiphenyl-4-aldehyde	$COOEt.C_6H_4.C_6H_4.CH$:	128,000	256,000
157	4′-Carboxydiphenyl-4-aldehyde	$COOH.C_6H_4.C_6H_4.CH$:	32,000	128,000
176	4-α-Pyridylbenzaldehyde	(II)	64,000	32,000
172	5-Acetamidothiophen-2-aldehyde	(III ; R=CH_3CONH)	512,000	512,000
180	5-Methylmercaptothiophen-2-aldehyde	(III ; R=CH_3S)	16,000	16,000
156	Pyridine-3-aldehyde	$3\text{-}C_5H_4N.CH$:	8,000[2]	8,000[2]
173	Diphenyl-4-aldehyde S-methyl-*iso* thiosemicarbazone hydriodide	(IV).HI	256,000	1,024,000

[1] Partial inhibition up to 512,000. [2] Partial inhibition up to 128,000.

4-acetamido-benzaldehydes (No. 143 and 102) are the two most active derivatives reported by other workers. We found these to inhibit growth completely only at high concentrations (M/8000 to M/16,000) but to show partial inhibition, as noted also by Hoggarth *et al.* (1949), at much greater dilutions.

In contrast, diphenyl-4-aldehyde thiosemicarbazone (No. 130) had a high and sharp anti-tuberculous end-point (M/512,000) and was not antagonised by serum. Its 4′-substituted analogues (No. 155 and 169), and the thiosemicarbazones of the corresponding ketones (No. 164 and 165) were all of the same order of activity. The more soluble derivatives (No. 157 and II : No. 176) had reduced activity. The related pyridine-3-aldehyde derivative (No. 156) showed complete inhibition only at high concentrations (M/8000), but again partial inhibition extending through 4 tubes was noted. This compound has been reported to be active by Levaditi, Girard, Vaisman and Ray (1951). In view of the reported *in vivo* activity of Tibione (No. 102) it was of interest to examine the isosteric analogue in the thiophen series (III ; R = CH_3CONH ; No. 172). This showed marked anti-tuberculous activity, apparently superior to that of Tibione. However, the derivative (III ; R = CH_3S ; No. 180) showed little activity whereas the benzene analogue is reported highly active *in vivo* (Behnisch *et al.*, 1948).

Since the intact thiosemicarbazone group is claimed to be essential for activity, the retention of substantially unaltered activity by diphenyl-4-aldehyde-S-methyl-*iso*thiosemicarbazone (IV ; No. 173), an analogue of No. 130, was surprising.

TABLE II.—*Thioureas and Related Compounds.*

Highest dilution expressed as 1/molarity of the substance completely inhibiting growth.

Lab. ref. number.	Substance.	Formula.	Organisms and media: H37Rv serum synthetic medium.	Organisms and media: H37Rv synthetic medium.
123	Phenylthiourea	$Ph.NHCSNH_2$	<2,000	<2,000
126	α-Naphthylthiourea	$C_{10}H_7.NHCSNH_2$	2,000	2,000
124	β-Naphthylthiourea	$C_{10}H_7.NHCSNH_2$	4,000[1]	8,000[1]
125	9-Phenanthrylthiourea	$C_{14}H_9.NHCSNH_2$	<8,000	16,000
97	4-Diphenylylthiourea	$Ph.C_6H_4.NHCSNH_2$	64,000	128,000
127	4-Diphenylyl-S-methyl-*iso*-thiourea	$Ph.C_6H_4.N : C(SMe)NH_2$	8,000[2]	16,000[2]
133	N : N′-Diphenylthiourea	$Ph.NHCSNH.Ph$	<8,000	<8000
132	N : N′-Bis-4-diphenylylthiourea	$Ph.C_6H_4NHCSNHC_6H_4.Ph$	,,	,,
141	Ammonium N-4-diphenylyl-dithiocarbamate	$Ph.C_6H_4.NHCS.SNH_4$	,,	16,000
134	Ammonium N : N-diethyl-dithiocarbamate	$Et_2NCS.SNH_4$	2,000	8,000
142	2-Mercaptobenzthiazole	(V)	4,000	8,000

[1] Partial inhibition up to 128,000. [2] Partial inhibition up to 1,024,000.

Thioureas.

Table II consists of aromatic thioureas. Only 4-diphenylylthiourea (No. 97) which differs from the related thiosemicarbazone (No. 130) in the absence of the CH : N-moiety, showed a moderate activity (M/64,000) and the related 4-diphenylyl-S-methyl-*iso*thiourea (No. 127) exhibited partial inhibition over a wide range. Since it has been suggested (Carl and Marquardt, 1949) that thiosemi-

carbazones owe their activity to the ability to form chelate compounds it was of interest to examine a dithiocarbamate (No. 141) capable of forming a stable metal complex and structurally related to No. 130. This and its N : N-diethyl analogue (No. 134) and the related 2-mercaptobenzthiazole (No. 142 ; V) were inactive.

The inhibitory end-points for *Staph. aureus*, *Bact. coli* and *Ps. pyocyanea* were uniformly low (< M/4000) with all compounds listed and have not been recorded in Tables I and II.

In vivo *Activity*.

Because of the promising *in vitro* activity of the thiosemicarbazones (No. 130 and 172), these were tested *in vivo*. Intramuscular injection of each into tuberculous guinea-pigs in doses of 60 mg./kg. body weight for 60 days showed no marked healing of the lesions and they were not further examined.

SUMMARY.

A number of thiosemicarbazones containing the diphenyl structure, together with some related thioureas have been examined for anti-tuberculous activity *in vitro*. Although a number of these showed marked activity (*e.g.*, 0·5 μg. per ml. for No. 130), they were ineffective *in vivo*.

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THE TITRATION OF LANSING POLIOMYELITIS VIRUS IN HeLa CELL CULTURES

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DURING the course of a serological study of a Lansing poliomyelitis virus strain adapted to growth in HeLa cells (Scherer, Syverton and Gey, 1953), it became necessary to measure the viral potencies of infected tissue culture fluids. The method of infectivity titration in HeLa cell cultures was selected for this purpose, partly on account of the ease with which cultures could be prepared and partly in anticipation of the subsequent use of the technique with poliomyelitis viruses unadapted to rodents. The absence of published reports on the reliability of this titration method, however, necessitated preliminary work designed to indicate the reproducibility of the test. Experiments were therefore carried out to compare the dose-response curve of the Lansing virus-HeLa cell system with that of the well investigated Lansing virus-mouse brain system, and thereafter to estimate the reproducibility of titrations performed in the virus-tissue culture system. This paper gives an account of the techniques employed and discusses the implications of the results obtained.

MATERIALS AND METHODS

Virus

The mouse-adapted Lansing poliomyelitis virus strain was obtained from Dr. Kingsley Sanders in 1954. After ten mouse brain passages in this laboratory HeLa cell cultures were inoculated with an aqueous suspension of spinal cord from a paralysed mouse infected intracerebrally. Adaptation occurred readily and thereafter the strain was passed serially in HeLa cell cultures. Pools of infected tissue culture fluids from the sixth and ninth HeLa cell culture passages were stored in 0·5 ml. volumes at −70° and −20° respectively.

HeLa cells

A single line of HeLa cells was used throughout. Initially the stocks were propagated by the technique of Syverton, Scherer and Elwood (1954) in stationary bottles measuring 55 mm. × 20 mm., but later their growth medium was replaced by one composed of human serum 10 per cent, five per cent solution of lactalbumin hydrolysate 5 per cent, tryptic meat broth 5 per cent, chick embryo extract 2 per cent and Earle's solution 78 per cent. This gave equally good results with the great advantage that only one-fourth the quantity of human serum was required. Using seeds of 1 million cells suspended in 10 ml. of this medium, bulk cultures of cells were grown in " Pyrex " babies' feeding bottles. Considerable economy of labour was thus achieved as one such bottle provided sufficient cells to seed one hundred of the small bottles. Sulphur-free rubber bungs were used.

Virus titrations in HeLa cell culture

The HeLa cells used for titrations were obtained by the trypsinisation of stock cultures. Suspensions adjusted to contain 100,000 cells per ml. were prepared in the growth medium in current use and dispensed in 1·0 ml. volumes in small bottles. After two days' incubation

vigorous cell growth was established and the cultures were ready for use. The growth medium was then removed from each culture and the cells washed thrice with Earle's solution at pH 7·2–7·4. Two ml. of maintenance medium were then added followed by an inoculum of 0·1 ml. of the appropriate virus dilution previously prepared in tenfold steps in 10 per cent fowl serum-saline. In the earlier experiments a maintenance medium was used consisting of bovine aqueous humour 50 per cent, rabbit serum obtained from young animals 10 per cent, chick embryo extract 1 per cent and Earle's solution 39 per cent. This maintained the cultures for eight days provided that a 50 per cent replacement with fresh medium was carried out on the fifth or sixth day. After the eighth day non-specific changes in the cells made reading of the virus titrations unreliable. Theoretically, the longer the observation period used to determine titration end-points the better but, in practice, it was found that, whilst the specific cytopathogenic effects of minimal doses of virus sometimes occurred after the sixth day, they never occurred after the eighth day. An eight day observation period was therefore adopted for routine titrations and various media recommended by other workers were tested with a view to obviating the necessity of replacement. The one adopted and still in use consists of fowl serum 10 per cent, five per cent lactalbumin hydrolysate 5 per cent and Earle's solution 85 per cent. This maintains cells in good condition for eight days without any replacement being necessary and 50 per cent replacement on the eighth day permits observations to be continued for a further six days if this is desired for any special reasons.

Virus titrations in mice

Serial tenfold dilutions of virus were prepared in 10 per cent fowl serum-saline. White mice three to four weeks old were used, 0·03 ml. of the appropriate virus dilution being injected into the left cerebral hemisphere. Mice dying within 24 hr. of inoculation were discarded. Death or paralysis occurring during the following 28 days was deemed to be due to virus infection.

EXPERIMENTAL

Comparison of Virus Titration in HeLa Cell Cultures and in Mice

In order to estimate the susceptibility of HeLa cell cultures to infection with Lansing poliomyelitis virus, titrations of both the sixth and the ninth passage pools were performed. Ten HeLa cell cultures were inoculated with serial virus dilutions prepared immediately beforehand in tenfold dilution steps. Each culture was observed daily for eight days for the cytopathogenic effects of the inoculum. These first appeared on the third day in cultures receiving the largest inocula and progressively later in cultures receiving the smaller. Parallel titrations of the two virus pools were performed in mice. The results are given in Table I and in the Figure which facilitates comparison of the dose response

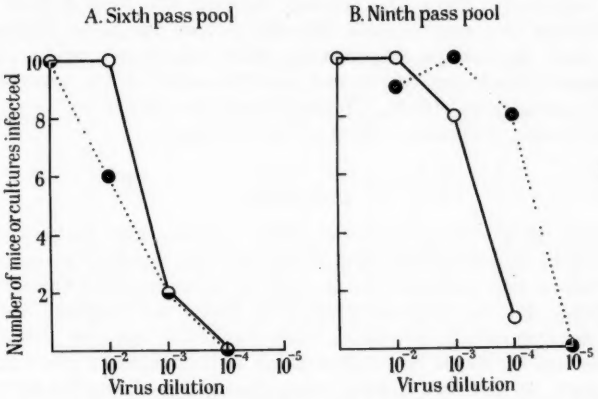
TABLE I.—*Comparison of the Susceptibility of HeLa Cell Cultures and Mice to Virus Infection*

	Sixth passage pool.				Ninth passage pool.				
Virus dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Infected cultures	10	10	2	0	10	10	8	1	—
Dead or diseased mice	10	6	2	0	—	9	10	8	0

Each figure shows the number of cultures destroyed or mice killed out of a total of ten.

curves. The dilution difference between 100 per cent affected and 100 per cent unaffected lies virtually within a 100-fold range in both HeLa cell and mouse

titrations. The phenomenon of an occasionally very resistant mouse (or maladministered inoculum), as shown in graph B, is without a counterpart in the tissue culture system. In spite of the small numbers of animals used in the



FIGURE—Comparison of dose-response curves of HeLa cell cultures and mice to infection with Lansing poliomyelitis virus.

Graph A = Sixth pass pool.
„ B = Ninth „ „
Unbroken lines = Titrations in HeLa cell cultures.
Broken „ = „ „ mice
Ordinates = Number of mice or cultures infected.
Abscissae = Virus dilution.

mouse titrations, these results are remarkably consistent with those reported elsewhere using much larger groups (Morgan, 1947) and they show that the culture-adapted Lansing strain had retained the full mouse pathogenicity of the parent strain. The advantages of the HeLa cell titration method are therefore not dependent on a virus adaptation affecting its virulence for mice.

Replicate Titrations of Lansing Poliomyelitis Virus in HeLa Cell Cultures

A consideration of the slopes of the dose response curves of HeLa cell cultures and mice to inocula of Lansing virus indicated that titrations in tissue culture would probably be more reproducible than titrations in mice. Replicate virus titrations were done in HeLa cell cultures using the ninth passage pool fluid. The method of preparing virus dilutions in tenfold steps was retained but, in order to permit a reasonable number of comparative titrations to be performed at one time, only five bottles were inoculated with each dilution. Three series of titrations were performed, each on a different day and each consisting of five replicates. The five sets of virus dilutions used for each series were prepared on each occasion from a freshly thawed ampoule of stock virus and held at + 4° until used as soon as possible after preparation. The HeLa cell cultures employed were grown from different serial subcultures of the same stock line. Fowl serum-lactalbumin hydrolysate maintenance medium was used in each experiment. Each culture was observed daily for cytopathogenic change. In the first series of

five titrations observations were continued for twelve days after inoculation, a medium change being effected on the eighth day. As no cytopathogenic change became evident for the first time in any tube of this series after the seventh day, the second and third series were observed for eight days only so that no medium change was required. From the records showing the first day on which cytopathogenic change was seen in each affected culture the titres expressed by each titration on each day of the experiments were calculated, using the method of moving averages (Thomson, 1947) and a nomogram for the interpolation of 50 per cent end-points (Lim, 1954). These results are shown in Table II, together with the daily mean values and standard deviations.

DISCUSSION

Estimations of the dose-response curves of mice and HeLa cell cultures to graded inocula of a culture-adapted strain of poliomyelitis virus show that the reactions of tissue cultures tend to be slightly more uniform than those of mice. This is probably due to the homogeneity of HeLa cell cultures and the facility with which an accurately measured virus inoculum may be introduced into a culture. The use of HeLa cell cultures for poliomyelitis virus titrations seems likely, therefore, to provide greater reproducibility of results than the use of similar numbers of mice, even when, as in the experiments described, the virus to be quantitated is a rodent-adapted strain which has undergone only limited tissue culture passages. A very great advantage of the HeLa cell culture technique is the much shorter time required for completion of a virus titration. Although we adopted an observation period of eight days in order that no occurrence of cytopathogenic effect due to virus should be missed, a considerably shorter period is used by many workers. Ample justification for this is provided by a consideration of the titres reached daily by the replicates of each series. Each titration is seen to increase in parallel with the others in its series with remarkably little irregularity or disparity throughout the entire period of observation. An analysis of the variance of the standard deviations calculated for each day of observation of each series shows no significant differences in value between either days of observation or titration series. In the light of these findings the four-day observation period commonly employed has much to commend it, though it is manifestly unsuitable for the estimation of small amounts of a slowly growing virus strain. In contrast to this, titrations in mice require an observation period of at least three weeks in order to include late deaths from the specific infection.

The results reported here are inadequate for the development of a precise test of significance applicable to the comparison of the titration end-points of two different virus preparations, even when the titrations are done in parallel under identical conditions. Table II shows that the maximum difference recorded between two titrations of the same series on any day of observation was 0.8 log. For convenience in practice a difference of 1·0 log between two titration end-points has therefore been adopted as the minimum indication of a real difference in potency of the fluids titrated. In titrations using a Leon virus strain in human embryo tissue cultures Lahelle (1955) found that there were no significant differences between the results of titrations of the same virus pool performed on different days. The analysis of variance of eighth day results given in Table III shows that this certainly cannot be claimed for the Lansing virus-HeLa cell system.

TABLE II.—*Virus Titrations in HeLa Cell Cultures : Daily 50 per cent End-points over Period of 8 Days*

	Titration.	Days after inoculation. 1+2	3	4	5	6	7+8
Series I	1	0	2·29	2·50	3·09	3·50	3·50
	2	0	2·29	2·91	3·09	3·29	3·29
	3	0	2·29	3·29	3·50	3·91	3·91
	4	0	2·63	3·09	3·70	3·91	4·09
	5	0	2·70	3·09	3·31	3·50	3·50
	Mean	–	2·45	2·96	3·27	3·61	3·66
	SD.	–	0·202	0·301	0·297	0·281	0·324
Series II	6	0	2·91	3·70	3·91	3·91	3·91
	7	0	2·70	3·91	4·29	4·70	4·70
	8	0	3·50	3·70	4·50	4·70	4·70
	9	0	3·30	3·70	4·29	4·50	4·70
	10	0	2·91	3·31	3·91	4·29	4·50
	Mean	–	3·06	3·66	4·18	4·42	4·50
	S.D.	–	0·326	0·218	0·261	0·317	0·342
Series III	11	0	3·50	3·91	4·50	4·50	4·50
	12	0	3·29	3·70	4·50	4·50	4·50
	13	0	3·29	3·50	4·09	4·50	4·50
	14	0	3·50	4·09	4·70	4·70	4·70
	15	0	3·29	3·50	4·70	4·70	4·70
	Mean	–	3·37	3·74	4·49	4·58	4·58
	S.D.	–	0·115	0·257	0·249	0·109	0·109

Figures are logs of 50 per cent end-points.

The variability of titration results within each of the three series can be accounted for by the error of the test, but the variability between the three series points to the operation of some unknown variable factor. Either a change in cell susceptibility or an irregularly occurring constituent of fowl serum used in the maintenance medium may be responsible.

TABLE III.—*Analysis of Variance of Eighth Day Titres Shown in Table II*

Item	Sum sqs.	D.F.	Mean sq.	V.R.	P.
Total	3·5657	14			
Series	2·6141	2	1·3075	16·488	0·001
Replicates	0·4841	4	0·1210	2 071	0·2
Error	0·4675	8	0·0584		
Replicates + error	0·9516	12	0·0793		

The use of five cultures per tenfold dilution step has been used in the replicate titrations recorded. By this arrangement it has been found possible to achieve reasonable reproducibility of results without unduly limiting the number of comparative titrations which can be done in a day. On occasions, greater accuracy has been achieved both by increasing the number of cultures inoculated with each dilution and by reducing the dilution intervals. Both modifications, also a combination thereof, have given satisfactory results ; the former, however,

generally commends itself because fewer technical operations are involved. Any degree of titration accuracy desired is theoretically obtainable by the methods described, but the unknown variable operative on different days implies that titres cannot be expressed as absolute values but only in relation to the titre of a standard preparation or other virus fluid determined by an equally extensive titration done in parallel.

SUMMARY

HeLa cell cultures have been found to respond more uniformly than mice to graded inocula of Lansing poliomyelitis virus.

Replicate titrations performed in parallel using five cultures per tenfold dilution step showed that the method is capable of giving reproducible results within an experimental error of less than 1·0 log dilution and that this holds true for readings taken from the third to the eighth day after inoculation.

Considerable variations in titre occurred when titrations of the same virus pool were performed on different occasions. The variable factor responsible for this is unknown.

Methods of increasing the accuracy of the results are discussed.

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THE EFFECT OF TYPHOID-PARATYPHOID A AND B VACCINE ON THE ANTIGENIC EFFICIENCY OF TETANUS TOXOIDS OF DIFFERENT QUALITY

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A NUMBER of workers have reported that the response of persons or animals to immunisation with certain antigens is increased when these antigens are mixed with another before injection. Barr and Llewellyn-Jones (1953) suggested, however, that some such claims were based on slender evidence. They reported experiments showing that the effects of immunisation with a combined prophylactic may depend on the relative proportions of the components and the dosage. They showed that the addition, to tetanus toxoid, of a particular batch of typhoid-paratyphoid A and B vaccine increased the response of guinea-pigs to the tetanus toxoid at one level of dosage: when the dose was increased 2·5-fold, no synergic effect was observed. When twice as much of the same batch of vaccine was added to the same tetanus toxoid, the response of guinea-pigs to the toxoid was not increased at either of two levels of dosage. The addition of a different batch of T.A.B. vaccine to the same tetanus toxoid effected a significant reduction in the responses to the toxoid. Further evidence of the effect of total dosage of a combined prophylactic on the response of guinea-pigs to one of its components was provided by Barr (1954). Groups of guinea-pigs were immunised with diphtheria-tetanus prophylactics containing, per ml., 60 Lf of diphtheria toxoid and 6, 12 or 25 Lf of tetanus toxoid. At one level of dosage, the diphtheria antitoxin responses of the animals receiving the 25 Lf tetanus toxoid preparation were significantly lower than the responses of those receiving the 6 or 12 Lf preparations. When however the dosage of these same prophylactics was increased four-fold, there was no significant difference between the responses of the groups: the excess tetanus toxoid no longer depressed the diphtheria response.

Further work has now been carried out to investigate the effect of varying amounts of T.A.B. vaccine on the antigenic efficiency of tetanus toxoid. In addition, since earlier results suggest that the quality of an antigen might influence the response of animals to another antigen mixed with it before injection, this point has been investigated using toxoids which showed a marked difference in antigenic efficiency per Lf.

EXPERIMENTAL

It has long been recognised that qualitative differences exist among batches of toxins or toxoids of the same potency as measured by tests for combining power with antitoxin. Further evidence of differences in quality of tetanus toxoids has recently been provided by Greenberg (1955) who, using a single dose method, investigated batches prepared by a number of different laboratories. We have found, at these Laboratories, that a rough qualitative screen-

ing of tetanus toxoid can be effected by titrating the antitoxin content of sera from guinea-pigs taken 10 days after the second of two injections, each 0·2 Lf of tetanus toxoid, separated by an interval of 28 days. By following this procedure it was possible to select two batches of tetanus toxoid, one of which (an autolysate toxoid) was a very poor antigen. This batch, which was considerably the more potent, in terms of Lf per ml., was diluted in a suitable broth to the same potency, 8 Lf per ml., as the other toxoid. In view of the well known unreliability of the flocculation test for tetanus toxoids, the Lf values were checked by a total combining-power test in mice by mixing varying volumes of toxoid with 0·2 unit of antitoxin and subsequently adding the L +/10 dose of toxin to all mixtures at a definite time before injection.

A concentrated suspension of typhoid-paratyphoid A and B vaccine was added in varying amounts to portions of each of the toxoids. The final batches of T.A.B.T. (T.A.B. vaccine + tetanus toxoid) contained respectively, per ml., $2{\cdot}5 \times 10^8$ *Salmonella typhi* in batch *a*, 5×10^8 in batch *b*, 10^9 in batch *c* and 2×10^9 in batch *d*, together with half these quantities each of *S. paratyphi* A and B. Ten antigens were thus available for comparison, *viz.*, the two unmixed toxoids and four batches of T.A.B.T., of varying vaccine content prepared with each. These antigens were tested by a two-dose method at several levels of dosage. Groups of 15 guinea-pigs of the same stock and of comparable weight were injected subcutaneously with the appropriate dose diluted to 1 ml. in normal saline : the injection was repeated 28 days later and the animals were bled by cardiac puncture 10 days after the second injection. The individual sera were titrated for tetanus antitoxin by the mouse method (Glenny and Stevens, 1938) : the results are recorded in International units (1950).

RESULTS

Results with toxoid A

Table I shows the responses of guinea-pigs to immunisation with the better quality tetanus toxoid and the four batches of T.A.B.T. prepared with it. Results for all five preparations are shown at each of three levels of dosage, and for the toxoid only and one preparation, A*c*, at other levels. When doses of only 0·01 ml. were given, a few guinea-pigs receiving toxoid A produced less than 0·01 unit of antitoxin per ml.: these results are not included in Table I.

The results obtained when two doses, each 0·02 ml., were injected, are of little interest, and it appears that the addition of the vaccine did not greatly influence the response of the animals to the toxoid. It is, however, evident that on increasing the dosage to 0·04 ml., the presence of the vaccine exerted a beneficial effect on the response to the tetanus toxoid. Thus the means of the logarithms of the antitoxin titres of sera from guinea-pigs injected with either A*a* or A*b* were significantly higher than the mean from those injected with toxoid A alone ($P = < 0{\cdot}01$ in both cases). In addition, as the amount of vaccine injected was increased, from *a* to *d*, the magnitude of the responses showed a progressive decrease and the mean logarithmic titre of animals injected with preparation A*a* was significantly higher than the corresponding titres of those injected with either A*c* or A*d*, containing 4 and 8 times as much vaccine ($P = 0{\cdot}05$–$0{\cdot}02$ and $< 0{\cdot}01$).

On further increasing the dosage to 0·1 ml., the responses were again in reverse order to the amount of vaccine present in the preparation. Whereas the increased dosage had a marked effect on the response to toxoid only and an appreciable effect on the responses to A*a* and A*b* (containing the smaller amounts of vaccine), animals which received A*c* or A*d* responded no better to a dose of 0·1 ml. than to 0·04 ml. The effect of proportion of vaccine injected on the response to the tetanus toxoid was thus greater at a dosage of 0·1 ml. than at 0·04 ml., and the responses of guinea-pigs immunised with A*b* were significantly higher than those of the

TABLE I.—*The Distribution of Tetanus Antitoxin Titres of Guinea-pigs 10 Days after the Second of Two Injections of Tetanus Toxoid A, and Batches of T.A.B.T., Aa, Ab, Ac and Ad, Containing Increasing Amounts of T.A.B. Vaccine*

Dose injected (ml.).	Material.	Over / Under	Number of guinea-pigs with tetanus antitoxin titres (units/ml.) 1·0 2·0	2·0 5·0	5·0 10	10 20	20 50	50 100	100 200	Geometric mean.
0·02	A		1	4	7	1	1	—	—	5·86
	A*a*		2	4	3	3	2	1	—	7·53
	A*b*		—	2	3	7	3	—	—	11·8
	A*c*		1	2	5	6	1	—	—	8·32
	A*d*		2	3	8	2	—	—	—	5·33
0·04	A		—	2	3	7	2	—	—	11·1
	A*a*		—	—	—	2	7	5	1	41·0
	A*b*		—	—	1	5	4	4	1	30·0
	A*c*		—	2	0	4	6	2	—	20·3
	A*d*		—	1	1	6	7	—	—	17·8
0·10	A		—	—	2	2	10	1	—	24·5
	A*a*		—	—	—	1	4	8	2	56·2
	A*b*		—	—	—	—	9	6	—	43·6
	A*c*		—	—	1	8	5	1	—	19·7
	A*d*		—	—	2	5	8	—	—	19·8
0·20	A		—	—	—	5	9	1	—	25·5
	A*c*		—	—	1	5	8	1	—	23·1
0·50	A		—	—	—	1	12	1	—	31·6

T.A.B. vaccine content of A*d* was double that of A*c*, four times that of A*b* and eight times that of A*a*.

animals which received A*c* or A*d* ($P = < 0{\cdot}01$ in both cases): this was not so at the dosage level 0·04 ml. where differences in response between A*b*, A*c* and A*d* were not significant. The responses to A*a* and A*b* were again significantly higher than the response to toxoid A ($P = < 0{\cdot}01$).

The marked increase in response which occurred on increasing the dosage of toxoid A from 0·04 to 0·1 ml. suggested that still larger doses might stimulate the production of antitoxin titres of the same order as those produced by A*a* and A*b* in doses of 0·04 or 0·1 ml. In contrast, the absence of any difference in response between groups injected with 0·04 or 0·1 ml. of either A*c* or A*d* suggested that a "saturation point" might have been reached, so that again no effect would be caused by further increasing the dosage.

The responses of groups of guinea-pigs to two doses, each 0·2 or 0·5 ml. of toxoid only, and to doses of 0·2 ml. of A*c* are included in Table I. These results confirm the suggestion that dose-response relation had already reached a saturation point with A*c* at a dosage of 0·04 ml., but it would appear that a saturation point to tetanus toxoid A had been approached at a dosage of 0·1 ml.

It is possible that doses of 0·2 ml. or more of A*a* and A*b* would have evoked higher antitoxin titres than doses of 0·1 ml. This was not investigated, for it is evident from Table I that the difference in response at dose levels 0·02 and 0·04 ml. was far greater than the difference at dose levels 0·04 and 0·1 ml. Saturation response point was therefore probably approached with a dose of 0·1 ml. It is

however interesting to note that the mean logarithmic titres of the sera of guinea-pigs injected with 0·1 ml. doses of A*a* and A*b* were both significantly higher than that of the group injected with 0·5 ml. of toxoid alone ($P = < 0{\cdot}01$ and 0·05–0·01). This suggests that the addition of another antigen, *in suitable proportions*, can raise the maximum level of response to a toxoid above that which it is capable of stimulating when injected alone.

The effect of increasing the dosage of tetanus toxoid while maintaining a constant dose of T.A.B. vaccine can be seen by studying the responses of groups of guinea-pigs injected with two doses of 0·01 ml. of A*c*, 0·02 ml. of A*b* and 0·04 ml. of A*a*. In addition to these three groups, others were injected with 0·1 ml. of a new preparation containing 2/5 of the T.A.B. content of A*a* and with 0·2 ml. of another preparation containing 1/5 of the T.A.B. vaccine content of A*a*.

Dose-response curves are shown in the Figure where the means of the logarithms of the antitoxin titres of guinea-pigs have been plotted against the logarithms of the doses injected. Curve 1 shows the responses to tetanus toxoid A, curve 2 to T.A.B.T. A*c*, and curve 3 shows the effect of increasing tetanus toxoid while maintaining a constant dose of T.A.B. vaccine.

For each curve the logarithms of the mean antitoxin titres show an approximately linear relation with the logarithms of the dose injected at the lower levels of dosage. An abrupt change occurs in the shape of the curves, which appear to flatten out. The slope of the rising part of curve 1 is less steep than the corresponding slopes of curves 2 and 3, and the abrupt change in curves 2 and 3 occurs at a lower level of dosage than in curve 1. Curve 1 flattens out at a slightly higher response level than curve 2 : there is a suggestion that the responses in curve 1 were still showing a slight but insignificant rise beyond the apparent flattening. The maximum mean antitoxin titres stimulated by these preparations can be estimated from the curves to be 28–29 units per ml. in animals immunised with tetanus toxoid, about 22 units in those given T.A.B.T. preparation A*c* and about 37 units when constant vaccine-increasing tetanus toxoid was used.

The figures for toxoid A in Table I provide an example of the familiar reduction in standard deviation of log titres of groups of animals, which occurs on increasing dosage. This phenomenon is, however, better illustrated in the results on preparations given in Table II.

While a good deal of time has been directed by many workers to preparing dose-response curves in experiments involving single-dose methods, scant attention has been paid to dose-response relations in the two-dose method. This is unfortunate because sound evidence that the results of single-dose methods of assay reflect efficiency in human immunisation is lacking at the present time. It appears that so long as secondary stimulation is necessary in human immunisation comparisons of the efficiency of prophylactics should be made by two-dose methods (Glenny, 1931 ; Barr and Llewellyn-Jones, 1951). Single dose methods give no indication whatever of the degree of potential immunity developed.

Toxoid B

Comparisons between the poor quality toxoid B and the four preparations of T.A.B.T., B*a*, B*b*, B*c* and B*d* were not so detailed as those made between toxoid A and the corresponding batches of T.A.B.T. A*a*, etc. The antigenic quality of toxoid B was such that, even at a dosage of 0·04 ml., some guinea-pigs in each

group produced less than 0·01 unit of antitoxin per ml. (the lowest level tested) and the scatter in all groups was considerable at this dosage. At lower dosage levels the majority of the animals in all groups produced less than 0·01 unit of antitoxin per ml.

The responses of guinea-pigs to two doses, each 0·1 or 0·2 ml. of the five preparations, are shown in Table II. In no instance did the presence of the T.A.B. vaccine exert a synergic effect. Indeed, in complete contrast to results with toxoid A, the log mean response of animals to toxoid B, at the 0·1 ml. dose, was significantly higher than the responses of the groups injected with B*b*, B*c* or B*d* ($P = 0{\cdot}05$–$0{\cdot}02$ in each case). On increasing the dose to 0·2 ml., the scatter of titres was much reduced, and the significance of the difference between the log means for toxoid B and either B*c* or B*d* was greater at this level of dosage ($P = < 0{\cdot}01$ in both cases): the response to toxoid B alone was also significantly higher than that to B*a* at this dosage, but owing to the high standard deviation of the B*b* group, the difference between the log means for this group and the toxoid group was not significant ($P = 0{\cdot}1$–$0{\cdot}05$).

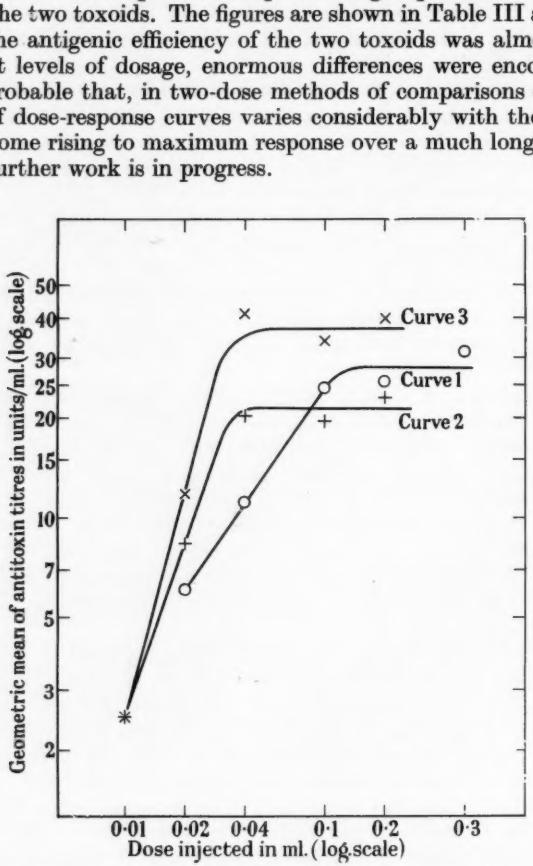
The actual amount of vaccine present in the preparations had no real effect on the response to tetanus toxoid B, in contrast with the results of toxoid A, where responses decreased progressively with increasing vaccine content.

This experiment was not extended to include higher levels of dosage apart from toxoid B, which was injected into a group of guinea-pigs in doses of 0·5 ml. Table II shows that the geometric mean titre increased by approximately 50 per cent on increasing the dose both from 0·1 to 0·2 ml. and from 0·2 to 0·5 ml. This rate of increase is considerably less than that for animals injected with doses of 0·02, 0·04 and 0·1 ml. of toxoid A: these responses can be seen in Table I, and in curve 1 of the Figure.

It is clear from Table II that the chief advantage gained by increasing the dose of all preparations was a reduction in the scatter of titres. The distribution of values is interesting. At a dosage of 0·1 ml. the scatter of titres was fairly considerable in all groups; the majority of animals had antitoxin values of 2 units or more per ml., but all groups showed a tendency to a tail in the distribution. When the dosage given was 0·2 ml. the scatter was greatly reduced, and only one obvious poor responder (in group B*b*) remained. The increase in geometric mean titre thus gives a false impression, particularly in group B*d*, of the improvement in response. The chief effect of increased dosage was to improve the titres of the very poor responders of the population, without altering to any great extent those of the majority. It is interesting to compare the individual responses of animals immunised with 0·1 and 0·2 ml. of B*d* (Table II) with those of animals that received doses of 0·02 and 0·04 ml. of either toxoid A or T.A.B.T. A*c* (Table I). Doubling the dose of B*d* increased the mean response 1·73-fold and greatly reduced the scatter, but doubling the dose of A or A*c* increased the response 1·89 and 2·44-fold, leaving the scatter practically unchanged but the titres distributed around a higher mean. The small scatter of titres of groups of animals injected with 0·2 ml. doses of B*c* and B*d*, and to a lesser extent of B*a*, compares with the distribution for A*d* at the 0·1 ml. dose level and suggests that no great increase in response would occur if larger doses were given.

It will be noted from Tables I and II that the geometric mean titres of guinea-pigs injected with either 0·2 or 0·5 ml. doses of toxoid B were approximately equal to those of guinea-pigs injected with the same doses of toxoid A. It is there-

fore of some interest to compare the responses of groups of animals to these and lower doses of the two toxoids. The figures are shown in Table III and it is evident that whereas the antigenic efficiency of the two toxoids was almost the same at the two highest levels of dosage, enormous differences were encountered at low dosage. It is probable that, in two-dose methods of comparisons of antigens, the configuration of dose-response curves varies considerably with the quality of the toxoid tested, some rising to maximum response over a much longer dosage range than others. Further work is in progress.



FIGURE—The antitoxin responses of guinea-pigs to two injections of tetanus prophylactics. Curve 1. Crude tetanus toxoid A. Curve 2. Toxoid A + 10^9 *S. typhi* + 5×10^8 *S. paratyphi* A and B per ml. Curve 3. Toxoid A + the same constant amount of *S. typhi* and *S. paratyphi* A and B at all levels of dosage.

DISCUSSION

As Jerne and Maaløe (1949) have pointed out, the maximal effect of a single dose of non-adsorbed diphtheria toxoid is low as compared with the maximal effect of alum-precipitated toxoid. A similar picture is seen in these experiments, in as much as the maximum effect of two doses of tetanus toxoid A, to which had been added relatively small amounts of T.A.B. vaccine, was greater than the maximum effect of the same toxoid alone. There is no reason to believe, however, that the vaccine acts as an adsorbent of toxoid. In an experiment made in collaboration

TABLE II.—*The Distribution of Tetanus Antitoxin Titres of Guinea-pigs 10 Days after the Second of Two Injections of Tetanus Toxoid B, and Batches of T.A.B.T., Ba, Bb, Bc and Bd Containing Increasing*

TABLE II.—*The Distribution of Tetanus Antitoxin Titres of Guinea-pigs 10 Days after the Second of Two Injections of Tetanus Toxoid B, and Batches of T.A.B.T., Ba, Bb, Bc and Bd Containing Increasing Amounts of T.A.B. Vaccine*

Dose injected (ml.).	Material.	Number of guinea-pigs with tetanus antitoxin titres (units/ml.). Over / Under	0·02 / 0·05	0·05 / 0·10	0·10 / 0·20	0·20 / 0·50	0·50 / 1·0	1·0 / 2·0	2·0 / 5·0	5·0 / 10	10 / 20	20 / 50	50 / 100	Geometric mean.
0·10	B		—	—	—	—	—	1	1	1	5	6	—	14·5
	Ba		—	—	1	0	0	1	1	2	4	6	—	10·2
	Bb		—	—	—	2	0	0	3	3	5	1	—	5·44
	Bc		—	—	—	—	—	2	4	5	2	2	—	6·17
	Bd		1	0	0	0	1	1	2	5	2	2	—	4·37
0·20	B		—	—	—	—	—	—	—	2	4	7	1	21·5
	Ba		—	—	—	—	—	—	2	5	7	1	—	9·71
	Bb		—	—	—	—	1	0	0	5	7	2	—	10·2
	Bc		—	—	—	—	—	—	—	9	4	2	—	10·4
	Bd		—	—	—	—	—	—	4	4	6	—	—	7·56
0·50	B		—	—	—	—	—	—	—	—	3	10	2	30·0

TABLE III.—*The Distribution of Tetanus Antitoxin Titres of Guinea-pigs 10 Days after the Second of Two Injections of an Average Tetanus Toxoid (A) and a Poor Quality Toxoid (B), of the Same Combining Power*

Dose.	Toxoid.	Number of guinea-pigs with tetanus antitoxin titres (units/ml.). Over / Under	— / 0·01	0·01 / 0·02	0·02 / 0·05	0·05 / 0·10	0·10 / 0·20	0·20 / 0·50	0·50 / 1·0	1·0 / 2·0	2·0 / 5·0	5·0 / 10	10 / 20	20 / 50	50 / 100	100 / 200	Geometric mean.
0·01	A		4	0	0	0	0	3	2	2	3	—	—	—	—	—	—
	B		14	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0·02	A		—	—	—	—	—	—	—	1	4	7	1	1	—	—	5·86
	B		14	0	0	0	0	0	0	1	—	—	—	—	—	—	—
0·04	A		—	—	—	—	—	—	—	—	2	3	7	2	—	—	11·1
	B		4	0	0	0	0	0	0	0	7	1	1	1	—	—	—
0·1	A		—	—	—	—	—	—	—	—	—	2	2	10	1	—	24·5
	B		—	—	—	—	—	—	—	1	1	1	5	6	—	—	14·5
0·2	A		—	—	—	—	—	—	—	—	—	—	5	9	1	—	25·5
	B		—	—	—	—	—	—	—	—	—	2	4	7	1	—	21·5
0·5	A		—	—	—	—	—	—	—	—	—	—	1	12	1	—	31·6
	B		—	—	—	—	—	—	—	—	—	—	3	10	2	—	30·0

with my colleague, Dr. A. E. Francis, no loss in potency was detected in a batch of tetanus toxoid tested at intervals for several days after the addition of T.A.B. vaccine : the supernatant liquid taken at varying times from other batches of T.A.B.T. has always been found to be of the same potency, within the error of the flocculation test, as that of the parent tetanus toxoid. The enhanced antigenic efficiency must therefore be due to some other effect or effects.

In a single experiment it was found that the tetanus antitoxin response of guinea-pigs to two doses of tetanus toxoid mixed with T.A.B. vaccine was significantly higher than the response of another group of guinea-pigs to the same doses of these two antigens injected into different sites. This observation requires confirmation, but suggests that T.A.B. vaccine may act as a local irritant and delay the absorption of the toxoid. If this is so, it is evident from the results in Table I that an excess of vaccine mixed with toxoid A may cause *undue* irritation, which no longer has a beneficial effect on the response to toxoid. Such a suggestion would not however account for the failure of the same T.A.B. vaccine to enhance the response of guinea-pigs to toxoid B. There are several other points to consider.

It has been shown (Glenny, 1931) that the response of horses to hyperimmunisation with certain toxins of the gas gangrene group may be approximately the same whether the horses are immunised with culture filtrates from one or from two distinct organisms simultaneously. Horses immunised with three distinct toxins usually gave reduced responses. There may be a limit to the total antibody content that can be reached, irrespective of the type of antibody. In the work quoted no higher titre was reached when only one toxoid was used rather than two, but this may have been due to gross over-neutralisation of the injected toxins or toxoids by circulating antitoxin. This could not occur in any single-dose experiments with prophylactics, but might occur in two-dose experiments involving large primary stimuli. Any conception of total antibody-producing capacity should probably include antibacterial as well as antitoxic bodies. When relatively small doses of mixed antigens are given a margin would exist between actual response and the full response of which the animal is capable. With greater dosage it might be expected that any increased response to one antigen would occur at the expense of the response to the other. The response of guinea-pigs to the T.A.B. vaccine in the experiments is unknown, but results of incomplete experiments, in which a mixture of two toxoids was used, suggest that this may in fact occur in certain circumstances.

In the work recorded in this paper relatively small amounts of T.A.B. vaccine had the effect of increasing the response of guinea-pigs to toxoid A : larger amounts of vaccine had no effect. The two preparations with the lower concentration of organisms may have been antigenically unbalanced, containing excess tetanus toxoid in relation to the T.A.B. vaccine content. If this were the case it might be that the increased response to the toxoid occurred at the expense of the response to the vaccine. The remaining two preparations containing larger proportions of vaccine may have been balanced, with neither component in sufficient excess to disturb the response of animals to either.

The response to toxoid B was not increased but was in general lowered by the addition of the same vaccine, in any of the quantities used. This may have been due to the fact that all the batches of T.A.B.T. produced from toxoid B were antigenically unbalanced. The optimum amount of vaccine necessary to prepare a balanced prophylactic may have been very small, less than that present in any of

the mixtures tested. The antigenic efficiency of this toxoid was very poor, and thus the T.A.B. vaccine may have been in excess in all preparations.

With the limited data available it is possible only to make tentative suggestions towards explaining the results. Work is now in progress using antigens composed of two toxoids : the antitoxic response to both components can be measured in such systems.

SUMMARY

Mixtures containing a constant amount of tetanus toxoid and varying amounts of typhoid-paratyphoid A and B vaccine were prepared and tested for antigenic efficiency by a two-dose method in guinea-pigs.

With one toxoid the tetanus antitoxin responses of animals to preparations of lower vaccine content were significantly greater, at high dosage levels, than that to the toxoid alone. No increased response was observed when preparations of higher vaccine content were injected, and the geometric mean tetanus antitoxin titres of the guinea-pigs decreased progressively as the proportion of vaccine present was increased.

Similar mixtures containing the same batch of vaccine and another batch of tetanus toxoid of poorer antigenic quality behaved in a different manner. The response to the toxoid alone was significantly higher than the response to most of the toxoid-vaccine preparations.

It therefore appears that the antigenic quality of a toxoid plays an important part in determining whether synergy or a reduced response will follow the injection of mixtures of the toxoid with another antigen.

I wish to thank Miss M. Llewellyn-Jones for performing some of the guinea-pig injections.

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THE CHEMOTHERAPY OF EXPERIMENTAL MOUSE TYPHOID WITH FURAZOLIDONE.

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THE antibiotics employed so far in the treatment of human enteric infections leave many patients still carrying the infecting organism at the end of prolonged courses of the drugs. When the antibiotic is withdrawn, they may multiply again, and clinical relapse, focal lesions or the chronic carrier state may supervene.

One factor contributing to the failure to eliminate the infection may be that the drug has only a bacteriostatic action. The fact that chloramphenicol is the best available antibiotic whereas bactericidal drugs such as streptomycin have very little clinical value suggests that this is not necessarily the only or even the main factor. Among others which may affect the efficiency of an antibiotic is the local environment of the organism within the body. The site of the infection, the number of organisms present and the changes in infected tissues may vary considerably during the course of the disease. Changes not existing a few hours after infection may later greatly affect the ability of the drug to reach or to eradicate the organism. Therefore, new drugs of possible value in the treatment of salmonella infections should be assessed for their capacity to kill the causative organism not only *in vitro*, but under appropriate conditions *in vivo*.

It is doubtful if any evidence of significance for the human disease can be obtained from studying the therapy of *Salmonella typhi* infections in any of the commonly used laboratory animals. *Salm. typhi* injected intraperitoneally in large doses into mice causes a disease of short duration with a " toxic " type of death in 2–3 days, or recovery with little tendency to chronicity or prolonged carriage (Ørskov and Kauffmann, 1936). Most of the organisms multiply at the site of the injection and are not widely disseminated (Bacon, Burrows and Yates, 1951): chemotherapy of such infection is more akin to *in vitro* experiments than to treatment of the natural infection in man.

In contrast to *Salm. typhi*, *Salm. typhi murium* is a natural pathogen of mice. It was shown by Ørskov and his colleagues (Ørskov, Jensen and Kobayashi, 1928; Ørskov and Moltke, 1928) that the experimental infection of mice with *Salm. typhi murium* has a course similar in many respects to that of human typhoid fever. Topley and his colleagues (Greenwood, Hill, Topley and Wilson, 1936; Lockhart, 1926) in extensive studies of mouse typhoid showed that recovery from the acute disease is usually associated with continuing infection, from which the animal may die after a chronic illness, or survive with persistent carriage of virulent organisms. *Salm. typhi murium* is closely related antigenically to *Salm. paratyphi B*, the commonest cause of human enteric fever in this country. A study

of the therapy of experimental mouse typhoid may therefore be useful in evaluating drugs of possible value in human salmonella infections.

The present paper reports an investigation of the *in vitro* activity of a new drug, furazolidone, against species of salmonella capable of causing human disease, and an assessment of the ability of the drug to cure mice systemically infected with *Salm. typhi murium*.

Furazolidone is a member of the nitro-furane group of drugs, shown by Dodd and Stillman (1944) to possess a wide range of antibacterial activity. Furazolidone (N-5-nitro-2-furfurylidene-3-amino-2-oxazolidone) was described by Yurchenco, Yurchenco and Piepoli (1953) and found to be effective in low concentrations against various gram-negative bacilli. Recently, Smith (1955) has reported that furazolidone is effective in the treatment of fowl typhoid caused by *Salmonella gallinarum*.

MATERIALS AND METHODS.

Many of the strains tested *in vitro* were recently isolated and identified by the Salmonella Reference Laboratory, Colindale. Others were derived from the National Collection of Type Cultures, and the strains of *Salm. typhi murium* were provided by Brig. J. S. K. Boyd of the Wellcome Foundation. All cultures were maintained on Dorset egg medium.

Furazolidone is only sparingly soluble in water. For *in vitro* tests, solutions were made each day by dissolving 10 mg. in 100 ml. broth by boiling, followed by Seitz filtration. It was later found easier to dissolve 10 mg. in 2 ml. dimethyl formamide, sterilise by boiling for 20 min., and to make further dilutions in broth. Dimethyl formamide has no antibacterial action in dilutions greater than 1/60. Solutions made by both methods gave identical results *in vitro*.

Sensitivity tests were performed by a tube-dilution method. The medium used was PLYP broth (Challice and Gorrill, 1954). The concentrations of furazolidone ranged from 10 to 0·05 μg./ml. Organisms to be tested were grown with continuous agitation for 3 hr. and diluted to give a final concentration of 5×10^3 organisms per ml. in the tube test. After stationary incubation at 37° readings were made at 24-hr. intervals up to 72 hr. A loopful of medium was plated from each tube showing no visible growth at 72 hr.

In all animal experiments a pure strain of white mice was used at weights of 22–25 g. The mice were shown by repeated tests to be free from natural salmonella infection. After inoculation all animals were kept in individual glass jars to obviate the risk of cross-infection. They were fed on a salmonella-free pellet diet with water *ad lib*, and inoculated intraperitoneally with organisms in the logarithmic phase of growth. A large batch of freeze-dried ampoules of each strain of *Salm. typhi murium* was prepared. For each experiment one ampoule was opened and seeded into broth. After overnight culture, a small volume was added to fresh broth and grown with continuous agitation for 2½ hr. The culture was washed and resuspended in phosphate buffer and standardised turbidimetrically. Bacterial counts were made on the dilution used for injection.

At various intervals after infection mice were anaesthetised with chloroform and exsanguinated by cardiac puncture; 0·1 ml. samples of blood were plated, and serial dilutions made in ice-cold buffer for bacterial counts. From each mouse the spleen, liver, kidneys and lungs were removed aseptically and washed in sterile buffer to remove surface contaminating bacteria. The whole organ (except the liver where only one lobe, approximately one-third the total weight, was taken) was crushed in glass tissue-grinders with measured amounts of cold buffer; from these tissue homogenates bacterial counts were made. The remaining homogenate was added to 10 ml. broth and incubated for 24 hr. The whole colon was excised and incubated in selenite F broth. After incubation, subcultures on MacConkey agar were made from the gut and from the homogenates of any organ showing slight or no growth on immediate plating. The organ bacterial counts were expressed numerically if counts greater than 100 bacteria per organ were obtained.

Furazolidone could not be administered satisfactorily by parenteral routes, since all available solvents were toxic to mice. The oral route was chosen. The required dose of material was administered in 0·2 ml. of a finely ground suspension in a 1 per cent aqueous solution of gum tragacanth, by oesophageal catheterisation with a blunted curved steel needle.

RESULTS.

Experiments in vitro.

Factors affecting sensitivity of Salm. typhi murium.

Continued incubation resulted in some decrease in the apparent sensitivity, usually a 2-fold (one tube) difference, or occasionally 4-fold. Tubes showing no visible growth at 72 hr. were sterile on plating. Readings were taken at 24 and 72 hr. in all tests to give a measure of the bacteriostatic and bactericidal concentrations of the drug. Variations in inoculum size from 10^6–10 organisms per ml. produced moderate changes in the apparent sensitivity of the organism, the 24-hr. reading varying from 5 μg./ml. with the largest inoculum to 0·625 μg. per ml. with the smallest. The 72 hr. levels varied from 10–1·25 μg./ml.

The effect of variation of temperature of incubation over a range of 22°–40° was not significant. Addition of 10 per cent serum to the medium had no effect on the sensitivity of the organism.

The growth of Salm. typhi murium *in varying concentrations of furazolidone.*

A 3-hr. culture was added in a final concentration of 5×10^3 organisms/ml. to 10 ml. quantities of broth containing different concentrations of furazolidone, and to a tube of broth not containing furazolidone. The tubes were incubated with constant agitation to ensure mixing and aeration in a water bath at 37° for 72 hr. At intervals, samples of 0·1 ml. were taken, appropriate dilutions were plated, and bacterial counts made. From tubes showing no visible growth, 1 ml. samples were also plated at 24, 30, 48 and 72 hr. (Fig. 1). Furazolidone has a rapidly bacteri-

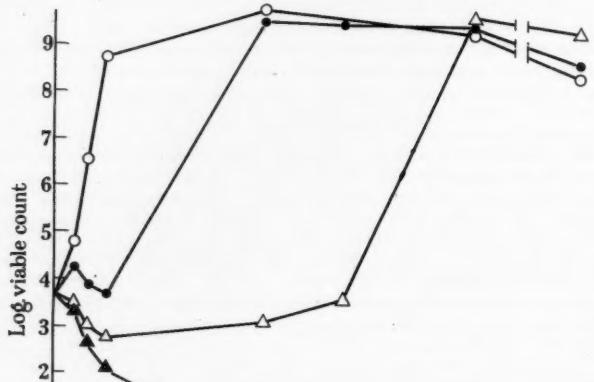

FIG. 1.—The effect of furazolidone in varying concentrations (μg./ml.) on the growth of *Salm. typhi murium in vitro.*

▲ 2·0 μg./ml. furazolidone.
△ 1·0 μg./ml. ,,
● 0·5 μg./ml. ,,
○ control : no ,,

cidal effect in a concentration of 2 μg./ml. Smaller concentrations of the drug produce some fall in the viable count, and appear to prolong the lag phase.

The sensitivity of various species of salmonella to furazolidone.

A total of 58 strains was tested (Table I). The behaviour of all the strains was similar, the 72-hr. inhibitory levels of furazolidone falling within the range of 2·5–0·625 μg./ml. Five streptomycin-resistant variants of *Salm. typhi murium* were as sensitive to furazolidone as were the streptomycin-sensitive parent strains.

TABLE I.—*The Sensitivity of 58 Strains of Salmonella in vitro to Furazolidone.*

Species.	Number of strains tested.	Minimum inhibitory concentration of furazolidone (μg./ml.). 24 hr. incubation.	72 hr. incubation.
Salm. typhi	11	0·625–1·25	1·25 –2·5
,, *paratyphi A*	4	0·625	1·25 –2·5
,, ,, *B*	8	0·625–1·25	0·625–2·5
,, ,, *C*	5	0·31 –1·25	0·625–2·5
,, *typhi murium*	15	0·31 –1·25	0·625–2·5
,, *enteritidis*	10	0·625–1·25	1·25 –2·5
,, *montevideo*	1	1·25	2·5
,, *seftenberg*	1	1·25	2·5
,, *newport*	1	0·625	1·25
,, *dublin*	1	0·625	1·25
,, *aberdeen*	1	1·25	2·5

Experiments in vivo.

The natural history of the experimental infection.

The intraperitoneal injection of 5×10^3 *Salm. typhi murium* causes a generalised infection which is fatal to almost all the mice within 28 days. The distribution of mortality is shown in Fig. 2, which is based on pooled data from 379 mice in several similar experiments. Deaths in the first 3 days were uncommon; there was a peak of mortality between 6 and 8 days after infection, and after 14 days only 30 mice (8·9 per cent) remained alive. By the 28th day there were only 9 (2·3 per cent) survivors: these were killed and found to be carrying small numbers of organisms (100–500) in the liver and spleen.

The course of the disease following similar infection was followed by killing groups of survivors at each day of the infection, and estimating the bacterial counts of each organ and of the blood. Within 16 hr. of inoculation the liver and spleen always contained small numbers of organisms. Their bacterial counts increased rapidly between 16 and 48 hr., blood cultures became positive, and positive cultures were obtained from kidney and lung. Between the 2nd and 4th day of infection the bacterial counts ceased to rise or fell slightly, but the fall was not maintained and counts of blood and all the organs rose progressively. Fig. 3 shows the sequence of events in the spleen, in a series of 63 mice.

The Effect of Furazolidone in Early Infections.

Forty mice were inoculated as above, and left for 24 hr. to allow establishment of infection in the liver and spleen. Furazolidone was administered in a single

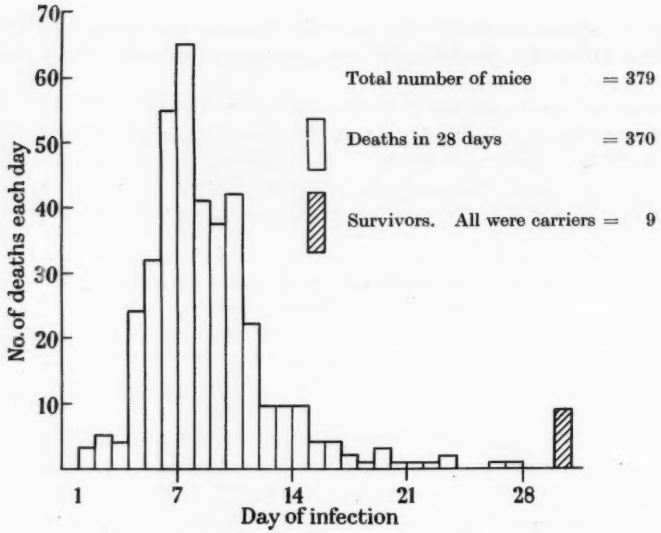

FIG. 2.—The distribution of mortality after infection with *Salm. typhi murium* (5×10^3 bact. I.P.).

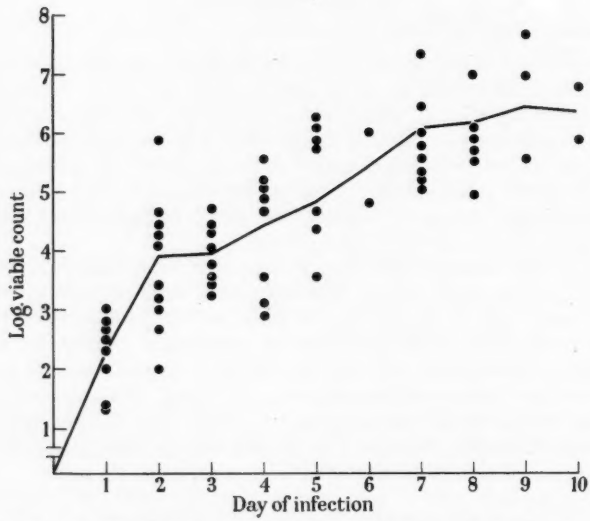

FIG. 3.—The distribution of spleen bacterial counts at each day of mouse typhoid infection (5×10^3 bact. I.P.).

● Bacterial count of the spleen of each mouse sacrificed.
—— Trend-line of bacterial multiplication based on the mean count at each day.

daily dose of 100, 50, 25 or 10 mg./kg. body wt. for 10 days, to groups of 5 mice. Twenty mice were left untreated. By the last day of treatment 17 of the 20 control mice had died. There were no deaths in the treated groups. The surviving animals were killed 24 hr. after the last dose, when no detectable concentration of the drug remained. From Table II it can be seen that the course of the infection was profoundly altered by furazolidone. The two higher doses had eradicated the infection in 6 of 10 animals. With lower doses all mice were carriers, but the bacterial counts in spleen and other organs were far lower than in the untreated controls, and blood cultures were sterile.

TABLE II.—*The Effect of Various Dosages of Furazolidone on the Mortality of Mouse Typhoid when Treatment is begun 24 Hours after Infection.*

Daily dose in mg./kg.	Number of deaths during the treatment period.	Spleen counts of each survivor 24 hr. after the last dose.				
100	0/5	+	−	−	−	−
50	0/5	1,100	320	+	−	−
25	0/5	3,000	100	+	+	+
10	0/5	1,500	2,500	4,000	9,000	+
0	17/20	3,300,000	550,000	10,000,000	..	..

− = Negative culture of homogenised spleen.
+ = Positive culture, but < 100 organisms per spleen.

The Effect of Furazolidone in Fully Established Infections.

Forty mice were infected ; 20 were left untreated. Groups of 5 of the remaining mice were given 10 daily doses of furazolidone 100 mg./kg. beginning 24 hr., 4 days, 7 days or 10 days after inoculation. The untreated mice were all dead by the 11th day of infection. No deaths occurred among mice treated from the 1st, 4th or 7th days after infection. Of the mice treated from the 10th day, two which were obviously ill when treatment began died on the 3rd day of treatment. The other three survived until the end of the course of furazolidone. All surviving mice were killed 24 hr. after their last dose of drug (Table III). Although treatment from the first day was effective in eradicating the infection, two of the five mice treated from the 4th day and all five treated from the 7th day remained carriers. The spleen counts, however, were much lower than would be expected in untreated survivors at this stage of the disease.

TABLE III.—*The Effect of Furazolidone* (100 *mg./kg./day*) *when Treatment is started at Various Stages in the Course of the Infection.*

Duration of infection (days) when treatment started.	Deaths in the 10-day period of treatment.	Numbers of survivors infected.	Spleen count of each survivor killed 24 hours after last dose.				
1	0/5	0/5	−	−	−	−	−
4	0/5	2/5	1,000	2,000	−	−	−
7	0/5	5/5	40,000	47,000	+	+	+
10	2/5	2/3	2,500	+	−	..	..

All of 20 untreated mice were dead by the 11th day of infection.
− = Negative culture of homogenised spleen.
+ = Positive culture, but < 100 organisms per spleen.

The Effect of Furazolidone on the Chronic Carrier State of Mouse Typhoid.

Mice infected with the strain of *Salm. typhi murium* S2/446 used in the foregoing experiments rarely survive to become chronic carriers. Reducing the dose merely leads to delayed deaths, and an injection of even 10 organisms causes approximately 50 per cent mortality in 28 days. The chronic stable stage of carriage could however be achieved without excessive losses of animals by infecting with a strain of reduced virulence.

From the parent strain of *Salm. typhi murium* S2/446 was derived a " one-step " mutant resistant to 5000 μg./ml. of streptomycin. *In vitro*, this mutant grew slightly more slowly than the parent in all media, but was otherwise unaltered. *In vivo*, injection of the mutant in doses of 1000 organisms resulted in less than 20 per cent mortality in repeated experiments, but all survivors were carriers and small numbers of organisms persisted in the liver and spleen of all animals for up to 10 weeks after inoculation. Of forty mice so inoculated, there were 32 apparently healthy survivors 21 days after inoculation; 16 of these were treated daily for 10 days with furazolidone 100 mg./kg. The remaining mice were left untreated. All the mice were killed and examined 24–48 hr. after the last dose. All but one of the untreated mice were carriers. Eight of the 16 treated mice continued to harbour the organism, despite a course of treatment which is sufficient to eradicate early infections with either the virulent or the mutant strain. The bacteria recovered from each of the treated mice were tested for furazolidone sensitivity. In no case had furazolidone resistance been acquired.

The Fate of Surviving Mice after Withdrawal of Treatment.

It seemed of interest to assess the effect of furazolidone on the mortality and carriage-rate from mouse typhoid in larger groups of animals, and to observe the fate of mice following the completion of schedules of treatment known to leave carriers. Individual caging of all animals excluded carriage or relapse due to cross-infection.

Four groups each of 20 mice were infected with 5000 organisms of the virulent strain. The first group was treated with furazolidone 100 mg./kg./day beginning 24 hr. after infection. The second group was given the same daily dosage beginning on the 5th day of the infection. The third group was treated with 25 mg./kg./day beginning 24 hr. after infection. Treatment in all three groups was continued for 10 days. The fourth group was left untreated, and all were dead by the 17th day of infection. The fate of the three treated groups during the period of treatment and after withdrawal of the drug is shown in Table IV. All mice were kept for 28 days after the last dose, and survivors were then killed and examined as usual.

TABLE IV.—*The Incidence of Bacterial Carriage and of Relapse in Infected Mice Treated with Furazolidone.*

Duration of infection when treatment started (days).	Daily dose in mg./kg.	Deaths under treatment.	Deaths in 28 days after treatment.	Number of carriers 28 days after treatment.
1	100	1/20*	0/19	3/19
1	25	0/20	0/20	19/20
5	100	2/20	3/18	12/15

20/20 untreated control mice were dead by the 17th day of infection.
* Death with signs of furazolidone toxicity. All cultures sterile.

The findings confirmed that when the dosage of furazolidone was reduced or there was delay in beginning treatment, eradication of the infection was not achieved in most cases. However, the treatment had modified the outcome of the disease. On the low dosage begun 24 hr. after infection, there were no deaths during treatment. All the animals survived the period of observation, although 19 of the 20 mice remained carriers. When treatment was delayed two mice died during the course of therapy. Of the survivors, three died between the 4th and 10th day after the last dose of furazolidone. The remaining 15 mice survived to the end of the period of observation. Twelve of these mice were carriers. One mouse treated with 100 mg./kg./day died with no evidence of infection. This was thought to be a toxic effect of furazolidone.

All organisms recovered from mice dying from the disease or killed after 28 days were tested for sensitivity to furazolidone. Of the 39 strains tested, 28 were as sensitive as the parent strain, 8 showed a 2-fold decrease, and 3 (all survivors) a 4-fold decrease in sensitivity.

The Effect of Furazolidone on the Course of the Infection.

In several related experiments, two groups of 20 mice were infected and one of the groups was treated with furazolidone. Each day, 24 hr. after the previous dose of the drug, two of the treated and two of the untreated mice were killed and organ bacterial counts were made. These counts were expressed numerically if more than 30 bacteria per organ were present. The untreated mice were all obviously ill from the 6th day of infection. Those sacrificed on the 8th, 9th and 10th day were moribund. Spontaneous deaths limited the total number sampled.

In general, when treatment with 100 mg./kg. was begun 24 hr. after infection, bacterial dissemination was prevented by the drug. Blood cultures and cultures of kidney and lung were sterile in all treated mice. The bacterial counts of liver and spleen were no higher on the second than on the first day of infection. No positive culture was obtained from any animal after the third or subsequent doses of the drug. When treatment with 100 mg./kg./day was delayed until the fifth day of the infection, the effect of the first few doses of drug greatly modified the findings in the treated mice. No positive blood cultures were obtained after the third or subsequent doses. Kidney and lung counts fell progressively, and were negative in all sampled mice after the fifth dose. Bacterial counts of liver and spleen appeared to fall sharply from levels of over 10^4 at the onset of treatment to less than 100 by the third dose, at a time when bacterial counts in these organs were rising progressively in the untreated mice. Despite this initial fall, the counts in both organs remained substantially the same in all animals subsequently tested despite continuing treatment. All organisms recovered were sensitive to furazolidone. These findings are illustrated graphically in Fig. 4 by plotting the spleen counts in mice sampled on each day. When mice were treated with 25 mg./kg./day beginning 24 hr. after infection, the spleen and liver counts rose during the first few days of treatment, but the number of organisms in the blood, kidney and lung remained at low levels. From the third day of treatment, the liver and spleen counts ceased to rise, and fell slightly towards the end of the course. The sequence of events in the spleen is illustrated graphically in Fig. 5. All organisms isolated from the mice were sensitive to furazolidone.

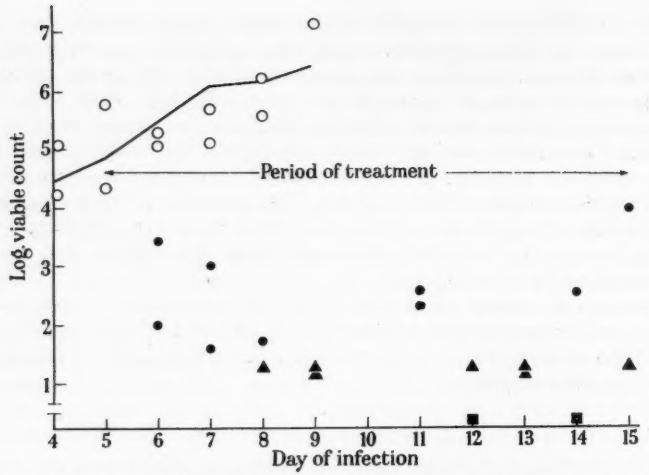

FIG. 4.—The effect of furazolidone (100 mg./kg./day) on the course of mouse typhoid in the spleen. Treatment withheld until the 5th day of disease.

○ Untreated mice.
● Treated mice count > 30 bact./spleen.
▲ " " " < 30 " "
■ " " sterile.
—— Average trend of multiplication in the spleen. Based on Fig. 3.

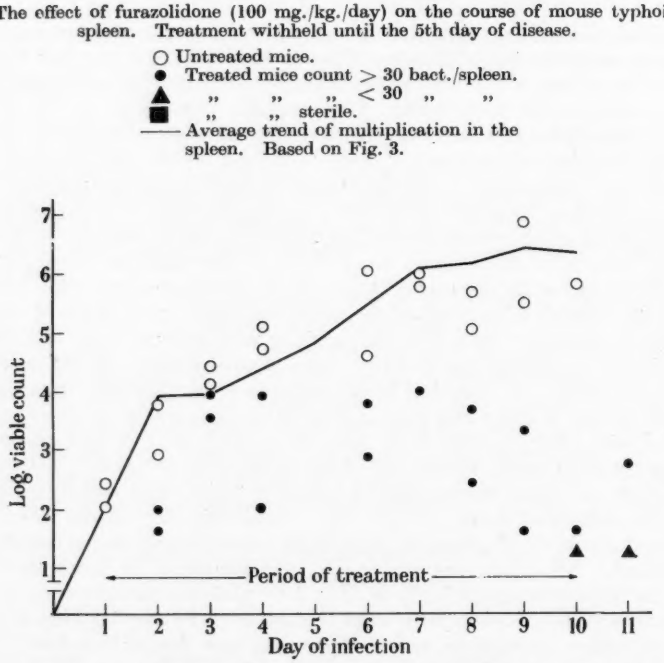

FIG. 5.—The effect of furazolidone in low dosage (25 mg./kg./day) on the course of mouse typhoid in the spleen.

—— Average trend of multiplication in the spleen. Based on Fig. 3.
○ Untreated mice.
● Treated mice count >30 bact./spleen.
▲ " " " < 30 " "

The Toxic Effects of Furazolidone.

Furazolidone was administered in single oral doses from 100 mg./kg.–1 g./kg. to groups of normal mice. There were no deaths nor illness. Similar single doses ground finely with gum tragacanth were given by the peritoneal or intravenous route. There were no deaths and all animals appeared healthy when killed and examined four days later.

Five groups of 5 mice were fed with furazolidone daily in doses of 100, 200, 300, 400, 500 mg./kg. for ten days. There was no illness in the first or second group. In the third group one mouse died and two were ill, in the fourth group two died and three were ill, and in the fifth group all were dead before the 7th dose had been given. The illness took a characteristic form. At first the animals showed choreiform movements and would roll over and over in the cages if disturbed. Subsequently they showed progressive paralysis, and died within 2–3 days. The disease did not appear clinically to resemble rolling disease of mice (Findlay, Klieneberger, MacCallum and Mackenzie, 1938), and was presumed to be a toxic effect of the drug.

DISCUSSION.

Provided that all animals are strictly segregated, the course of experimental mouse typhoid can be predicted for the rate of bacterial multiplication in each organ, for the total mortality in a 28-day period and for the average time to death. The few natural survivors from infection with 5000 organisms, which is approximately 500 LD_{50}, are all carriers. By reduction of the infecting dose or by substituting a strain of modified virulence mortality is reduced, but all surviving mice are carriers. The number of organisms in liver and spleen persists virtually unchanged for 4–10 weeks after infection.

Antibacterial agents may be assessed in such a model infection for three characteristics—their ability to reduce or delay mortality, their ability to eradicate the infection and prevent carriage, and their effect on the incidence of relapse in carriers after withdrawal of treatment. These aspects of the drug can be considered in relation to the phase of the infection at which treatment is begun. The three stages chosen are comparable with (1) the incubation period, with foci of infection in the tissues : (2) the fully established systemic infection, with bacteraemia : (3) the chronic stage of stable carriage.

Furazolidone was shown *in vitro* to be active in low concentrations against 11 species of salmonella. It was bactericidal in concentrations of 2–4 times the minimum inhibitory concentration, and its efficiency was slightly reduced by large increases in the size of the inoculum.

When furazolidone was tested in the experimental infection, the result of early treatment with large doses conformed to the findings *in vitro*. The infection was rapidly eradicated from all tissues in most animals.

Despite reduction of the daily dose or delay in starting treatment, furazolidone still had an effect on mortality, only those mice dying which were acutely ill when treatment began. Most of the survivors were still carriers, but the course of the disease had been retarded : infection was confined almost entirely to the liver and spleen, and the bacterial counts were low and comparable with those found normally only in the few chronic stable carriers one month or more after infection.

The failure of high doses of furazolidone to eradicate fully established infections

may partly be due to differences in the size of the bacterial population exposed to the drug. The total number of organisms in each organ was shown to rise considerably during the course of the disease. This does not seem, however, to be the whole explanation. In infection of 5 days' duration treatment rapidly eradicated bacteria completely from all organs except the liver and spleen. In these organs, the bacterial count fell rapidly to a level no higher than that normally seen within 24 hours after infection, yet continued treatment produced no further fall. In the chronic stable disease caused by the streptomycin-resistant variant of *Salm. typhi murium*, the infection when treatment began was localised to liver and spleen, and bacterial counts were comparable with those 24 hours after infection. Intensive treatment, however, failed to eliminate the infection in 8 out of 16 animals.

The failure to eradicate established infection is unlikely to be due to deficient absorption of the drug. The rapid sterilisation of kidney and lung and the rapid initial fall in spleen and liver counts were consistent with a bactericidal effect, and the sequence of events in the spleen was unlike the effect of a small dosage of the drug (Fig. 4 and 5). Residual infection after treatment is apparently not due to acquired drug resistance.

The reduced efficiency of the drug in the established disease may be due to the changed environment of the organisms. After 5–10 days, the liver and spleen contain small abscesses or necrotic foci in which there are intracellular organisms, presumably less accessible to the drug.

An interesting feature is that modification of the course of the disease by the treatment had a marked effect on the subsequent fate of the animals, despite the failure to eradicate the infection. Few mice relapsed when treatment was withdrawn. This suggests that the animals had acquired some immunity during treatment, sufficient to prevent multiplication of the carried organism, or to kill all but a small proportion of the bacterial population sequestered in tissue foci.

The efficiency of furazolidone in higher dosage could not be assessed because of the frequency of deaths from toxic effects. The evidence suggests that it is capable of exerting a profound effect on normally fatal systemic infections with *Salmonella typhi murium*. If further nitrofurane derivatives can be isolated of greater solubility and less toxicity than furazolidone they may be of value in the treatment of human salmonella infections.

SUMMARY.

Experimental mouse typhoid is a continued systemic infection, with a high mortality, and persistent carriage in survivors. Furazolidone is active *in vitro* against the causative organism, *Salm. typhi murium*, and other salmonellas causing human disease.

In the treatment of mouse typhoid furazolidone will eradicate the causative organism only when treatment with large doses is begun very early in the disease. Other schedules of treatment reduce mortality, but most of the survivors are carriers. These carriers show little tendency to relapse when treatment is withdrawn. In the chronic carrier state, furazolidone failed to eradicate infection in 8 of 16 mice tested.

The practicable dosage of furazolidone is limited by its toxic effects.

The failure of furazolidone to eradicate fully established infection is attributed to the relative inaccessibility of the organism in more chronic foci.

My thanks are due to Dr. Joan Taylor and to Brig. J. S. K. Boyd for cultures, and to Messrs. Menley & James (A. J. White) Ltd. for supplies of furazolidone. I am grateful to Mr. T. Norris for his care of large numbers of animals under single-cage conditions, and to Miss Italia S. Alderton for excellent technical assistance.

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RENAL NECROSIS IN MICE AFTER ACCIDENTAL EXPOSURE TO CHLOROFORM

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THE occurrence of acute necrosis of the convoluted tubules of the kidney of the adult male mouse after administration of sub-anaesthetic doses of chloroform was first described by Eschenbrenner (1945). In a later paper Eschenbrenner and Miller (1945) confirmed that adult females did not show this reaction. They also reported that male castrates were not susceptible but became so after treatment with testosterone.

Shubik and Ritchie (1953) reported a high death-rate from kidney necrosis in 55 adult male DBA mice accidentally exposed to a low concentration of chloroform vapour. Of an equal number of female DBA mice exposed under the same conditions, all survived.

Deringer, Dunn and Heston (1953) described a more serious accident in which a varied mouse stock was exposed to a low concentration of chloroform vapour. As a result of the exposure a high death-rate, again confined to adult males, was encountered in C3H mice. A smaller proportion of the exposed males of strains A and HR also died. Unweaned male C3H mice, C3H males bearing stilboestrol pellets and adult males of strains C57BL, C57BR/cd, C57L, and ST, were found to be free from kidney damage following the accidental exposure. These workers found acute necrosis in the kidneys of the affected males. They exposed male and female C3H mice for 1–3 hr. to a concentration of 5 mg. chloroform per litre in flowing air and confirmed the susceptibility difference between the sexes.

In spite of these reports, all from laboratories in the United States, the fact that adult male mice develop fatal kidney damage after exposure to very small concentrations of chloroform vapour appears not to be widely known. In the two accidents referred to, the rôle of chloroform in causing the deaths was not at first realised; and in the accident to be reported here considerable investigation was undertaken and much literature consulted before chloroform vapour was suspected of playing any part.

The object of this publication is to stimulate an awareness of the chloroform hazard in animal houses and to encourage the authors or editors of manuals which deal with the care of experimental animals to include a brief account of chloroform nephrosis in future editions of their works.

OBSERVATIONS ON THE ACCIDENTALLY EXPOSED MICE

Description of living conditions of the exposed mice

The animal house in which the present accident occurred consisted of three rooms intercommunicating by wide open portals and having a total cubic capacity

of 2040 cu. ft. In two of the rooms were housed several hundred CBA mice and a smaller number of mice of a heterozygous albino strain; the third room was used for servicing the animals. The stock of each strain comprised mice of all ages and both sexes. No epidemic of fatal infectious disease had occurred and no mice had been imported there for several years. The death-rate in normal animals had been low and until the accident to be described had shown no significant variation over a long period. All mice were fed on a standard compressed diet (the Thompson Cubed Stock diet) and given water *ad lib.* Ventilation was by two air extractors, one at either end of the house, both of which were operated continuously.

Examination of mice found moribund

In the eleven days immediately following the Christmas holiday period of three days, 28 adult male CBA mice were found dead or dying; all other mice including the albino males remained well. About two-thirds of the affected mice had been housed in the central one of the three rooms although only about one-quarter of the total mouse stock was housed there. In this room a glass-stoppered bottle of chloroform was kept for use in sacrificing unwanted mice. Moribund CBA males exhibited reduced spontaneous movement, a marked tremor, occasionally convulsions, and priapism. Much amorphous débris was seen in the uncentrifuged urine, but no cells or casts; the urine gave a dense white precipitate on mixture with an equal volume of 5 per cent sulphosalicylic acid (the urine of normal male CBA mice contains only a small amount of protein, giving only a faint opalescence by this test). The blood urea, examined in a few cases, was greatly raised and often approached 500 mg. per cent. The only gross finding on examination post mortem was a distinct white mottling of both kidneys, always symmetrical but varying in degree from mouse to mouse.

Examination of the surviving mice of the exposed stock

The appearance of the diseased kidneys was so striking that it could be easily recognised through the intact posterior abdominal wall after a small incision had been made in the skin of the back. By this means, a considerable number of the surviving CBA mice which appeared well were examined under ether anaesthesia for evidence of gross kidney disease. Of 127 CBA males examined, these being selected at random from all parts of the animal house, 72 per cent were found to have obvious kidney damage. Of 40 CBA females examined, half of which were from boxes containing affected male siblings, none showed evidence of kidney damage. In 82 of the male mice inspected at operation the precise age was ascertained. It will be seen from the Table that the incidence of gross renal damage in these mice was at least 78 per cent in all but the lowest

TABLE—*Evidence of Renal Damage in Male Mice of Different Ages after Assumed Accidental Exposure to Chloroform*

	Age group (days)				
	14–45.	46–75.	76–106.	107–136.	137–167.
Mice inspected	14	10	11	27	20
Mice with nephrosis	0	10	10	21	18
With nephrosis (per cent)	0	100	91	78	90

age group ; no mice of this group showed gross evidence of renal damage. This group contained exclusively mice which were unweaned at Christmas time.

Since deaths were confined to the CBA strain only a small number of male and female albino mice were examined for evidence of gross renal damage ; none was observed in these mice.

Histological appearances of the diseased kidneys

Histological examination of the diseased kidneys removed during the time when deaths were occurring revealed that the greater part of the proximal and distal convoluted tubules had been completely denuded of epithelium. There was no evidence of damage to the glomeruli, the capsules, the first or straight part of the proximal convoluted tubules, the collecting tubules, the renal papilla, or the renal pelvis (Fig. 1). Eosinophilic hyaline material was seen near the necrotic areas and in the lumen of the intact collecting tubules. In a few areas some increase of interstitial cells was seen, suggesting that some sublethal damage to the kidneys had been sustained on a previous occasion. The situation of the lesions, the absence of anasarca, and the presence of nitrogen retention in the affected mice were reminiscent of corrosive sublimate nephrosis in man.

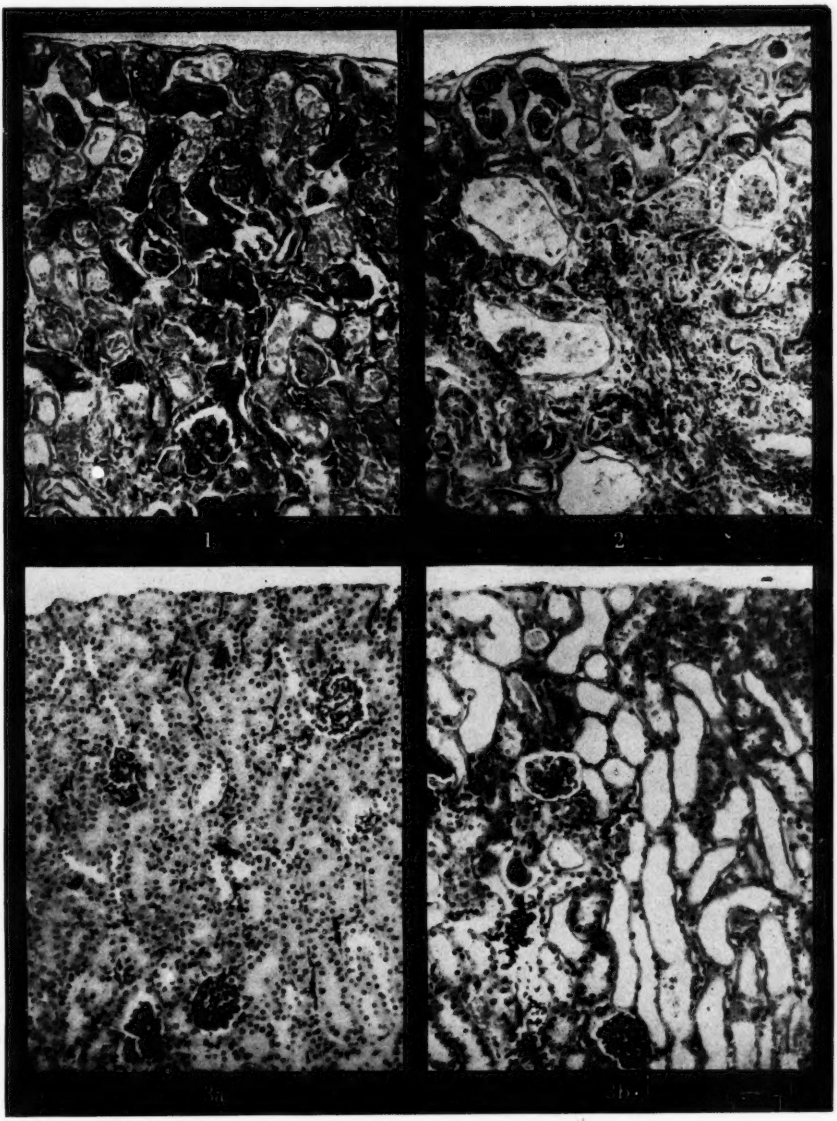
Several male mice which were found to have grossly diseased kidneys when inspected at operation early in January were killed 3–4 months later and their kidneys examined. At this later stage the kidneys were found to be granular and white and possibly slightly contracted. The histological appearance of these kidneys showed that the site of the originally necrosed tubules was replaced by fibrous tissue or by basophilic material suggestive of calcification ; there was no evidence of regeneration. Interstitial fibrosis was seen to extend into the outer part of the medulla, a zone which showed no damage at an earlier stage. Secondary changes were seen also in the glomeruli and capsules : where the changes were most advanced, some of the capsules appeared to have been obliterated entirely ; in less severe cases some of the glomeruli appeared atrophic, the capsular spaces were frequently dilated and there was usually flattening and some reduplication of the parietal layer of the capsular epithelium (Fig. 2). Since no damage to the capsules can be appreciated in the earliest stages it is considered that the late changes in these structures have been brought about by fibrous contraction in the tissue surrounding them. In spite of the fact that gross chronic changes of this nature are known to be present in a large number of male CBA mice that survived the initial wave of deaths ten months previously, only two late deaths have been attributable to kidney failure. There can be little doubt, however,

EXPLANATION OF PLATE

FIG. 1.—Kidney cortex of moribund male CBA mouse a few days after assumed accidental exposure to chloroform vapour. A high proportion of the convoluted tubules have undergone necrosis ; the glomeruli and capsules appear to be undamaged. H. & E. × 120.

FIG. 2.—Kidney cortex of male CBA mouse 5 months after assumed accidental exposure to chloroform vapour. The site of the convoluted tubules is occupied by basophilic débris or fibrosis. The capsular epithelium is flattened and atrophic, and the capsular spaces are enlarged. Heidenhain's iron haematoxylin. × 100.

FIG. 3.—a. Cortex of left kidney of normal male albino mouse, removed by operation, before exposure of the mouse to a low concentration of chloroform vapour. b. Cortex of right kidney of same mouse 24 hr. after the exposure. The epithelium of many of the tubules has undergone total necrosis. The black areas to the left of the Figure are stained coagulum in damaged tubules. Heidenhain's iron haematoxylin. × 120.



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that these damaged mice would show physiological abnormalities attributable to kidney damage. Their condition renders them unsuitable for any experiments in which the use of " normal " mice was specified.

EXPERIMENTAL STUDIES

After failure to demonstrate an infective agent by histological or bacteriological methods or by transmission experiments using organ extracts, attention was turned to the possibility that a chemical poison might be responsible. This had been suggested by the resemblance of the lesions to those which are seen in corrosive sublimate nephrosis in man.

The mouse drinking bottles had frequently been cleaned by soaking them in chromic acid solution. Since chromium salts have been known to cause renal damage male and female CBA mice were given to drink the highest concentration of chromic acid which they would take freely. No renal damage was found in mice given this fluid for one week.

Male and female CBA mice were also exposed in a closed container for about one hour on several occasions to a mixture of all volatile organic solvents (excluding chloroform) which, from their use in a chemical laboratory adjacent to the animal house, could have contaminated the atmosphere. This mixture consisted of approximately equimolecular proportions of ethyl alcohol, petroleum ether, benzene, hexane, ethyl acetate, pyridine, acetone and butyl alcohol. Although the mice became semi-conscious during their exposure, they quickly recovered on removal to fresh air and subsequently were found to be free from urinary or histological evidence of renal damage.

CBA males and females and albino males and females were exposed to small sub-anaesthetic concentrations of chloroform vapour by enclosing them for 2 hr. in a 6-l. desiccator containing an oxygen-enriched atmosphere and in which known weights of chloroform had been allowed to evaporate. The concentration of chloroform vapour attained in this desiccator at 29·5° was calculated on the assumption that the chloroform evaporated completely and became uniformly distributed. Of 16 CBA males and 12 albino males exposed under these conditions for 2 hr. to concentrations of chloroform vapour of 1·2 mg./l. in air or greater, all had extensive necrosis of the convoluted tubules of the kidney 24 hr. after exposure. The urine was found to contain numerous casts and large amounts of protein within 6 hr. of exposure. Similar results were obtained when the atmosphere in the desiccator was kept in circulation by an internal fan. Fig. 3 shows adjacent low power views (A) of the cortex of the left kidney removed by operation from a normal male albino mouse 24 hr. before exposure of the mouse to a sub-anaesthetic concentration of chloroform vapour, and (B) of the cortex of the right kidney from the same mouse 24 hr. after the exposure. The effect of lower concentrations of vapour could not be reliably ascertained by the method used and it is therefore not known whether the minimum kidney-damaging concentration is different for the males of the two strains. All of 8 CBA females and 8 albino females failed to show urinary or histological evidence of renal damage after exposure not only to low concentrations of the vapour but also to the much higher concentrations required to induce slight anaesthetic effects. A similar sex difference of susceptibility was demonstrated when mice of a third strain (heterozygous albinos) obtained from another laboratory were exposed

under similar conditions. The results described were repeated, using chloroform from two sources outside the laboratory, one of these being a bottle in clinical use in the hospital.

DISCUSSION

Evidence that the renal condition found in the stock mice was due to exposure to chloroform is necessarily circumstantial and rests on the following features: the resemblance of the lesions to those produced in mice by experimental exposure to chloroform as described by previous workers and as reported here; the resistance of both adult females and unweaned males; the failure of other chemicals which could have reached the mice to produce such lesions; and the fact that ample opportunities existed for contamination of the animal house atmosphere with chloroform.

Chloroform vapour could have contaminated the atmosphere of the animal house from three sources:

(1) On a few occasions mice had been sacrificed in the animal house by placing them in a chamber containing cotton wool moistened with chloroform.

(2) A glass-stoppered bottle of chloroform was kept in the animal house for use in killing unwanted mice.

(3) The drains of the animal house communicated with drains leaving sinks in an adjacent chemical laboratory. Used solvents, including chloroform, were occasionally poured down these sinks.

The extent to which each of these sources may have contributed to the exposure of the mice cannot be stated. A review of all the circumstances relating to the accident suggested that periodic discharge of vapour from the glass-stoppered bottle of chloroform kept in the animal house was the principal source of contamination of the atmosphere, and it is possible that the mice were exposed to a very low concentration of chloroform from this source over a long period. The occurrence of a wave of deaths over a fairly circumscribed period following the Christmas holidays is considered to be due to the fact that extra ventilation through the doors of the animal house, which are left open to the laboratory during the greater part of a normal working day, was in abeyance during the vacation period. At this time an exceptional rise in the chloroform vapour concentration may well have occurred. Unfortunately, it was impracticable to try to recreate the conditions that existed at the time of the accident and so test this hypothesis.

In the two similar accidents previously recorded a large proportion of the exposed susceptible mice were killed by the exposure. In the accident described here deaths were few compared with the number of susceptible mice whose kidneys were severely damaged. It is clear, therefore, that accidental exposure of a mouse stock to chloroform vapour could readily result in severe damage to the males without attention being drawn to their condition by the occurrence of unexplained deaths. In such circumstances mice with fairly gross damage to the kidneys might unwittingly be used for experimental purposes. It is probable that mice so damaged would react abnormally to the administration of drugs whose action is influenced by the state of renal function. On the other hand, it has been found that males with damaged kidneys do not appear to exhibit precocious sterility and can be usefully retained in the breeding stock.

A review of the previous reports of accidental and experimental exposure of mice to chloroform fails to reveal quantitative data which would enable the order of susceptibility of different strains to be determined. Eschenbrenner found that when 2×10^{-4} ml. of chloroform per gram body weight was administered in oil *per os* to adult males of strain A, half of the treated animals died. He used no other strain. Shubik and Ritchie found that strain DBA adult males developed fatal nephrosis after accidental exposure, whereas mice of strains A, C3H and C survived, although apparently equally exposed. They did not, apparently, investigate the surviving male mice of these exposed strains for the presence of sub-lethal kidney damage. Deringer *et al.* (1953) reported that adult males of strains C3H, A, and HR developed fatal nephrosis after accidental exposure, whereas males of strains C57BL, C57BR/cd, C57L, and ST appeared to be relatively resistant. In their experimental studies these workers confined their attention to strain C3H.

In the accident reported here, in which adult males of strain CBA and of a heterozygous albino strain were equally exposed to an unknown concentration of vapour, deaths were confined to the CBA strain and no damage was seen in the kidneys of the albinos. In experiments undertaken since the accident, however, adult males of the albino strain developed extensive nephrosis after exposure for < 2 hours to concentrations of vapour of < 2 mg./l. The escape of the albino males during the accidental exposure could not be explained by their having been segregated in a different part of the animal house from that occupied by the CBA mice, since affected CBA males were in all parts of it. Moreover, a fair proportion of the moribund CBA mice were in experimental boxes, in a situation where albino and CBA boxes were closely intermingled. It can only be assumed that the accidental exposure was to concentrations of vapour appreciably lower than the lowest concentration used experimentally, and that a strain difference of susceptibility existed at these lower concentrations.

Although the evidence suggests that the mortality of adult males from exposure to chloroform differs with the strain, there is no report of an adult male of any strain having been exposed to anaesthetic doses of chloroform vapour without showing some evidence of kidney damage by examination of the urine or of the kidney histology. Inquiries into the anaesthetic practices in an adjacent laboratory revealed that A.C.E. (a mixture of ethyl alcohol, chloroform and ether in the respective proportions 1 : 2 : 3) was used routinely for the anaesthesia of mice without untoward effects having been noticed. However, the male mice used in that laboratory showed gross histological and urinary evidence of kidney damage both when exposed to sub-anaesthetic doses of chloroform vapour and when kept under deep anaesthesia with chloroform for five minutes. Similar effects followed five minutes' anaesthesia with A.C.E. mixture. It must be assumed therefore, that the anaesthetic procedure in that laboratory had resulted in renal damage to the mice but this had escaped notice only because fatalities were rare.

A study of the toxicity of chloroform compared with other commonly used anaesthetics was recently reported by workers at the University of Wisconsin (Waters, 1951). Evidence of renal damage by examination of urea clearance, phenolsulphonephthalein excretion, alkali reserve, blood sugar, and blood non-protein nitrogen was looked for in 40 patients anaesthetised with chloroform. No difference was observed between these patients and an equal number receiving

various other anaesthetics. In dogs chloroform anaesthesia was associated only with a slight degree of cloudy swelling of the tubules. No other changes were seen although some dogs had been anaesthetised on six occasions. Two adult male rats were exposed in this laboratory to a chloroform vapour concentration ten times greater than that to which male mice are highly susceptible; neither rat showed any evidence of necrosis although moderately increased proteinuria resulted. Susceptibility to chloroform nephrosis therefore appears to be a species peculiarity of the mouse and provides a striking example of the grave limitations of mouse screening toxicity tests of industrial or therapeutic materials to which humans may be exposed.

Chloroform nephrosis in mice is of particular interest because it provides what seems to be an example of the hormone dependence of the reaction of a specific cell type to a simple organic substance. The results of experiments to be reported in a paper in preparation leave no doubt that chromosomal sex constitution has no appreciable influence on the susceptibility of the cells of the tubules to chloroform necrosis. For example, intact adult females became fully susceptible to chloroform nephrosis after treatment with an androgen. These conditions are such that the system might prove to be useful in studies relating to the influence of hormones on enzymes.

In considering the possible mechanism involved in the production of necrosis by the chloroform, it is not permissible, on the evidence available, to make the simple assumption that the necrosis is induced by the direct action of chloroform on cells conditioned by the direct action of androgenic hormone. The renal damage may be secondary to the effect of chloroform on organs remote from the kidney, and the influence of the hormone may then be exerted elsewhere. In this connection it should be mentioned that liver damage can regularly be demonstrated histologically in mice 24 hr. after exposure to very small doses of chloroform. The degree of damage, although very variable, shows no correlation either with hormone status or with the amount of renal damage. The liver lesions, unlike the kidney lesions, are transitory.

SUMMARY

After accidental exposure of a mouse stock to a low concentration of chloroform vapour a small proportion of the adult male CBA mice died with severe renal damage. Of the surviving adult CBA males 72 per cent were found at operation to have gross damage to the kidneys although their longevity and fertility proved not to be appreciably impaired. The renal lesions consisted of large areas of complete necrosis confined to the convoluted tubules. Gross secondary changes supervened in the kidneys of surviving affected mice after several months.

No unweaned CBA mice, adult female CBA mice, or any mice of an unrelated albino strain showed any evidence of renal damage although exposed under the same conditions as the affected mice.

Identical renal changes were induced in adult males of both the CBA and the albino strains by their exposure for 2 hr. to concentrations of chloroform vapour below 2 mg. per litre in air. Adult females of both strains showed no evidence of renal damage after exposure to these or any of the much higher concentrations that were tried.

I am indebted to the British Empire Cancer Campaign for grants during the tenure of which these observations were made, to Dr. R. M. Haslam for the blood urea estimations, to Mrs. D. Culliford for skilled technical assistance, and to Mr. F. Thomas, animal technician, for specially valuable assistance during the course of the investigation.

Since this paper went to press I have been informed by Dr. F. J. Aumonier of the Physiology Department of St. Bartholomew's Hospital Medical College that a similar accident happened in the new College Animal House between January 30 and February 3, 1956. During this period 91 male mice died. Of these 54 were of the A.B.C. strain, 28 were C3H and 9 white heterozygous. All were males.

The source of the chloroform was a killing box in which chloroform had been used on January 27. It was removed on February 1. The daily deaths from January 30 to February 3 numbered 22, 30, 33, 3 and 3; thereafter there were none.

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CHRONIC RENAL DISEASE IN RATS FOLLOWING A TEMPORARY DEFICIENCY OF POTASSIUM

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FOLLIS, Orent-Keiles and McCollum (1942) described lesions in the heart and kidneys of rats killed while on a potassium-deficient diet. The present experiment was carried out to discover what happened to these lesions if the rats were allowed to recover from their deficiency of K.

METHODS

The " experimental " group contained twelve young inbred black and white hooded rats of a strain obtained from the Lister Institute. Their starting weight was 180–250 g. and they were given a diet consisting of rice (87 per cent), casein (10 per cent) and arachis oil (3 per cent) to which were added cod liver oil, yeast, aneurin and a K-free salt mixture. The diet contained 0·014 per cent of K. For 10 days in every fortnight an ammoniated exchange resin (Zeokarb 225) was incorporated in the diet in the proportion of 20 g. resin to 100 g. diet. The resin removes K from the body; it also produces an acidosis, but the rats were able to correct their acidosis during the 4-day intervals when resin was not given, whereas the diet contained too little K to enable them to restore this deficit.

The first control group consisted of an equal number of animals who received the same food and vitamins, but they were given no resin, and their salt mixture contained enough K to provide 0·07 g. per 100 g. diet.

The second control group of 12 animals received the same diet with K in the salt mixture, but with the addition of 3 g. NH_4Cl per 100 g. diet for 10 days in every fortnight. This amount of NH_4Cl provided the same quantity of H^+ ions as the ammoniated resin, and the object of including this group of animals was to find out if any of the lesions found in the K-deficient animals could be attributed to an intermittent acidosis.

The resin and the experimental diets were given in the way described over a period of 8 consecutive weeks. During this period 6 animals in each group were killed, 4 of them after 6, and the remaining 2 after 8 weeks; they were killed in the intervals when animals were not receiving resin or NH_4Cl. All the animals remaining at the end of 8 weeks were given the stock diet of rat pellets. After 3 months on this diet 3 in each group were killed, and the remaining 3 in each group after they had had the diet for 7 months. The rats were killed under anaesthesia by bleeding them from the aorta. In some of the animals the percentage of K in the heart, the kidneys, the brain and the adductor muscles of the thigh was measured by the method of Mudge and Vislocky (1949). The hearts and kidneys were fixed in Heidenhain's *susa* fixative and stained with haematoxylin and eosin, van Gieson's, Weigert's elastic and picro-Mallory stain. During the course of the experiment the percentage of urea was estimated by the method of Lee and Widdowson (1937) in blood drawn from the rats' tails, and the urinary excretion of urea was measured when the animals were in metabolism cages by the same method.

RESULTS

Killed after 6 or 8 weeks

The rats deprived of K did not grow and even lost weight. The concentration of K in their serum was 2·5–3 mEq. per l., compared with 4 to 5 mEq. per l.

in both groups of control animals. The concentration of K in their muscle was reduced (Table). The analysis of other organs of the K-deficient rats showed that in the heart and kidney the loss of K was only slight and there was no depletion in the brain. The amount of urea excreted in the urine was similar in all groups, but the blood urea of the K-deficient rats rose to about 60 mg. per 100 ml. and in some animals much higher, while in all the others the blood urea remained at the normal level of about 30 mg. per 100 ml. The kidneys in all the K-deficient rats showed degenerative changes, mostly necrotic in type and usually limited to a short length of the ascending loop of Henle. The kidneys of both control groups were normal.

Killed 3 months after K deficiency had been corrected

These rats had restored their serum and their muscle K to normal, but their serum still contained about 60 mg. urea per 100 ml. There were degenerative changes, vacuolation, granularity of the cytoplasm, and pyknotic nuclei in most parts of the renal tubules. A further change, probably regenerative rather than degenerative, was the presence of clumps of up to a dozen closely clustered nuclei in the ascending loop of Henle. The kidneys of both control groups were normal.

Killed 7 months after K deficiency had been corrected

The blood urea remained about 60 mg. per 100 ml. in one rat; it had risen to 150 mg. in another and to 290 mg. in the third. The urine contained albumen and was of greater volume and lower specific gravity than in the control rats. All the control rats still had a normal blood urea, although, as frequently happens in older rats, they also now had some albumen in the urine. In the two rats with a high blood urea the hearts were larger than in both the groups of controls.

The kidney of the rats which had been made K-deficient had finely granular surfaces and were two to three times larger (Fig. 1) than the kidneys of the rats in the first control group (Fig. 2) or of those receiving NH_4Cl, which were all normal. The enlarged kidneys contained grossly dilated tubules, maximum in the proximal convoluted segment, with flattening of their epithelium and eosinophilic casts in their lumina (Fig. 3). Between the dilated tubules there were strands of reticulin and collagen, broader in the medulla than in the cortex (Fig. 3) containing occasional remains of degenerate tubules. Most of the glomeruli were atrophic and showed hyaline change. For the first time the kidneys of both control groups showed changes, namely, slight dilatation and flattening of epithelium in an occasional tubule, usually the proximal convoluted tubule. Lesions in the heart of the K-deficient rats were found but were not materially different from the description given by French (1952).

DISCUSSION

The experiments show that a temporary deficiency of K can lead to progressive renal damage in the rat. No claim is made that the final histological picture is peculiar to K deficiency. Apparently similar lesions appear in much older animals with advancing age (Saxton and Kimball, 1941), can be provoked by

giving a large excess of phosphate in the diet (Gough, Duguid and Davies, 1933; Duguid, 1933–34; MacKay and Oliver, 1935), or by the administration of desoxycorticosterone with an anterior pituitary extract (Selye, 1951), but in this case a deficiency of K may be responsible for the lesions (Durlacher, Darrow and Winternitz, 1942).

The experiments raise the question whether in man, as in rats, the renal lesions of K deficiency may lead to a progressive change and whether some of the renal lesions in so-called potassium-losing nephritis (Evans and Milne, 1954) may be the result and not the cause of the K deficiency (Conn, 1955).

SUMMARY

Rats were made K-deficient by diet and exchange resin for 6–8 weeks and they were then allowed to recover from their deficiency.

In the ensuing months lesions in their kidneys which were small in extent at the time of recovery became progressively more extensive.

After seven months, the rats had a high concentration of urea in their blood and their kidneys were enlarged with gross dilatation of tubules, and atrophy of most of the glomeruli.

The experiments show that a temporary deficiency of K can lead to progressive renal change in the rat.

We wish to thank Dr. A. M. Barrett for his help with the histology, and Mr. S. Patman for the photomicrographs.

TABLE—*The K Content of Muscle in mEq./100g. Fat Free Dry Muscle of K-Deficient Rats and Controls*

Duration of exp. diet in weeks.	K-deficient group.	Control groups. Normal salt mixture.	Normal salt mixture + NH_4Cl.
6	37.7	44.5	44.3
8	25.1	47.1	48.5
Duration of recovery stock diet in weeks.			
12	48.2	48.0	—
28	46.5	46.6	—

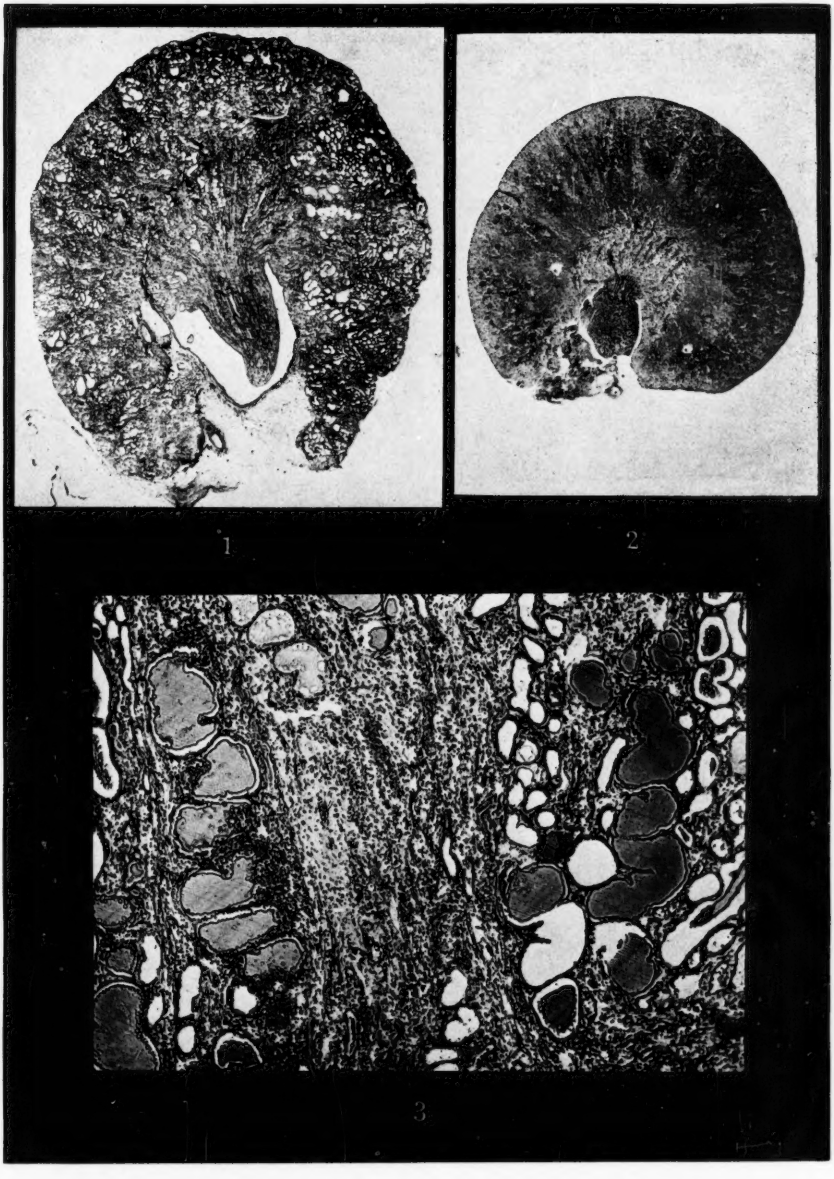
Figures represent means of determinations of 2 animals.

EXPLANATION OF PLATE

FIG. 1.—Enlarged kidney with dilatation of tubules from a rat on normal diet for seven months after an initial period of K-deficient diet. H. & E. × 8.

FIG. 2.—Normal-sized kidney of rat from first control group for comparison with Fig. 1, same magnification. H. & E. × 8.

FIG. 3.—Medulla of the same kidney as in Fig. 1 showing a band of reticulin and collagen fibres between dilated tubules with flattened epithelium. H. & E. × 73.



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CHANGES IN THE HISTOCHEMICAL APPEARANCES OF CHOLINESTERASE AT THE MOTOR END PLATE FOLLOWING DENERVATION

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THE histological changes which occur in nerve fibres following damage or section have been extensively studied by many workers. Tower (1935) described in great detail the cytological changes which occur in skeletal muscle following denervation. Few investigations, however, have been made into the effect of section of the motor nerve on the histology and histochemistry of the motor end plate. The present research was designed to study the effect of nerve section on the histochemical appearances of cholinesterase at the motor end plate and to relate the findings if possible to the increased sensitivity of denervated skeletal muscle to acetylcholine.

MATERIAL AND METHODS

Fourteen guinea-pigs, 7 of each sex, aged about 1 yr., were anaesthetised with ether and the right sciatic nerve was cut in mid-thigh, a portion approximately 1 cm. in length being removed. One animal of each sex was killed by a blow on the back of the neck 7, 11, 15, 19, 22, 28 and 45 days after nerve section. The denervated gastrocnemius was removed from each animal and at the same time the normal gastrocnemius was removed from the other leg to serve as a control. The muscles were fixed in 10 per cent formol saline for 6 hr. Frozen sections 50μ thick were cut from the anterior surface of each muscle and then washed in distilled water for 1½ hr. Sections from both muscles were then treated by the method of Koelle and Friedenwald (1949) and modified by Couteaux (1951) and Coërs (1953). Sections from each muscle were incubated for 30, 40, 50 and 60 min. Adequate control was obtained by always incubating together a denervated and a normal control muscle section for exactly the same length of time. The concentration and distribution of the cholinesterase were assessed by studying the copper sulphide precipitate at the motor end plate.

RESULTS

Cholinesterase Appearances in Control Muscles

The histochemical appearances of cholinesterase in all the control sections which had been incubated for the same length of time were practically identical. The intensity of staining was seen to be greatest in those sections which had been incubated for the longest period of time.

The staining of cholinesterase by this method showed the motor end plates in surface view as black irregular oval structures (Fig. 1), and in profile they were seen to be slightly raised on the surface of the muscle fibres.

The dimensions of the end plates showed considerable variation, the majority measuring about 25–35μ in length and about 19μ in width. These figures are in accord with those of Gerebtzoff, Philippot and Dallemagne (1954) for the cat and rat.

Examination of the sections under high power (Fig. 2) showed that the cholinesterase at the motor end plate is situated in the walls of gutters on the surface of the muscle fibres and is in the form of a series of horseshoe-shaped plates as described by Couteaux and Taxi (1952).

Cholinesterase Appearances in Denervated Muscles

The muscles which had been denervated for seven days showed that the concentration and distribution of cholinesterase at the motor end plate were similar to controls. In the muscles which had been denervated for 11 days the concentration of cholinesterase was slightly reduced compared with the controls, while in those which had been denervated for 15 days the concentration was further reduced and the gutters were less well defined, the edges being irregular. After denervation for 19 (Fig. 3) and 22 days, the cholinesterase was greatly reduced in amount, its distribution was uneven and the horseshoe-shaped plates were only visible in places. After denervation for 28 days the cholinesterase could only just be recognised as a faint shadow against the pale background of the muscle fibres (Fig. 4). No cholinesterase was found in the motor end plates of muscles denervated for 45 days.

Throughout the investigation the results in both sexes were identical.

DISCUSSION

Manometric estimations of cholinesterase activity in skeletal muscle after motor nerve section have been made by many workers. The results have however been confusing. Martini and Torda (1937) found that in the gastrocnemius of the rat a rapid diminution of cholinesterase occurred after nerve section. After 3 days the activity was reduced by 36 per cent and after 12 days by 74 per cent. Marnay and Nachmansohn (1937), working on the gastrocnemius of the guinea-pig, found that between the 8th and 28th days following nerve section the concentration of cholinesterase at the myoneural junction increased. Feng and Ting (1938) reported a diminution of the cholinesterase activity of about 20–30 per cent in the nerve-containing tibial portion of the toad sartorius between the 12th and 34th day after section of the sciatic nerve. No change was found in the nerve-free pelvic portion.

Nachmansohn (1938), reviewing his previous work on the concentration of cholinesterase in the gastrocnemius of the guinea-pig, suggested that the apparent increase in cholinesterase concentration after denervation was probably due to a considerable reduction in the volume of the denervated muscle fibres. He was of the opinion that while the volume of the muscle fibres decreases following denervation the number of motor end plates remained unchanged, at least during the period of the experiment (5 weeks).

Couteaux and Nachmansohn (1938) repeated the estimation of cholinesterase concentration in the guinea-pig gastrocnemius and on this occasion measured the relation between the volume of the normal and denervated muscle fibres. They found that 2 weeks after cutting the sciatic nerve the increase in the concentration of cholinesterase was approximately inversely proportional to the decrease in volume of the denervated muscle fibres. This indicated that the concentration of the enzyme at the end plates at this time was practically unchanged. Four

to five weeks after denervation the diminution in the volume of the denervated muscle fibres was greater than the increase in concentration of the enzyme. The concentration of the enzyme therefore seemed to be a little smaller than in the normal end plates. Couteaux and Nachmansohn (1940) were of the opinion that between the 3rd and 4th weeks following denervation the concentration of cholinesterase at the motor end plate diminished by about 30–40 per cent.

Stoerk and Morpeth (1944) pointed out that so far the manometric estimations of cholinesterase had generally been determined per 100 mg. of muscle tissue. Marnay and Nachmansohn had shown that the concentration of cholinesterase at the myoneural junction was many thousand times greater than in the surrounding muscle. Moreover it was known that the distribution of motor end plates in skeletal muscle is not uniform. Stoerk and Morpeth maintained that quantitative cholinesterase estimations were only valid if the entire muscle is extracted and the weight of the whole muscle taken into consideration since it could be assumed that for a given species the same muscle within certain variations contained a fixed number of motor end plates. By this procedure they found that in the rat the cholinesterase concentration of denervated muscle is reduced by one third and the reduction is not a function of time, for the concentration is the same 21 days after nerve section as after 2 days.

Sawyer, Davenport and Alexander (1950), using the skeletal muscle of the rat, guinea-pig and rabbit, reported that the concentration of cholinesterase was at first reduced following nerve section and later increased to considerably more than normal. They do not state whether the results were based on muscle weight or whether the cholinesterase in the whole muscle was extracted.

These findings taken together give a confused picture ; they do however show that unless extreme precautions are taken the results obtained by the manometric method are not valid.

The development of the histochemical technique for demonstrating cholinesterase at the motor end plate by Koelle and Friedenwald presented a new method for studying the problem. Kupfer (1951), working on the flexor muscles of the rat fore limb, reported that the cholinesterase-staining area at the motor end plate was decreased by about 72 per cent 39 days after denervation, the greatest decrease occurring during the first 16 days. They found, however, that the cross sectional area of the muscle fibres had decreased by about 77 per cent by the 39th day following denervation. They were thus unable to tell definitely whether any great change had occurred in the concentration of cholinesterase

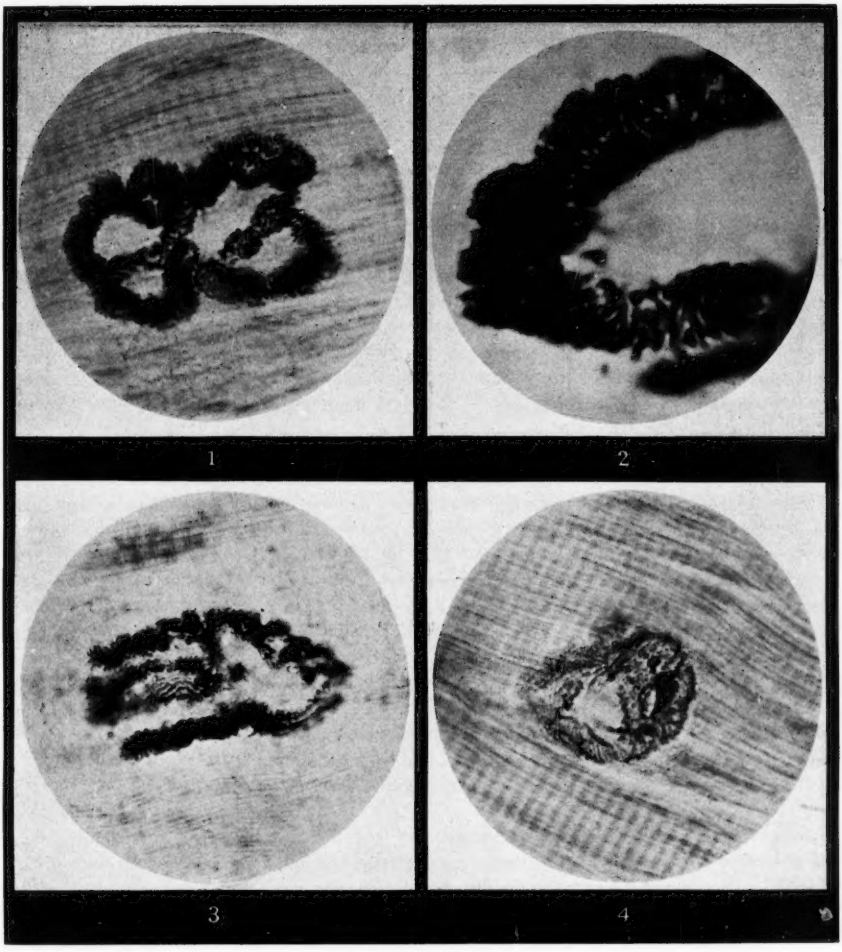
EXPLANATION OF PLATE

FIG. 1.—Photomicrograph of section of normal gastrocnemius muscle from adult male guinea-pig. Shows general distribution of cholinesterase at motor end plate. The cholinesterase appears black. Stained histochemically. × 1400.

FIG. 2.—Photomicrograph of part of one motor end plate of normal gastrocnemius muscle from adult male guinea-pig. Shows cholinesterase to be present in the form of a series of horseshoe-shaped plates. Stained histochemically. × 4000.

FIG. 3.—Photomicrograph of section of gastrocnemius muscle from adult male guinea-pig 19 days after section of sciatic nerve. Shows irregular distribution of cholinesterase at motor end plate and loss of normal configuration. Stained histochemically. × 1400.

FIG. 4.—Photomicrograph of section of gastrocnemius muscle from adult male guinea-pig 28 days after section of sciatic nerve. Shows cholinesterase at motor end plate as a faint shadow on the muscle fibre. Stained histochemically. × 1400.



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at the motor end plate. Moreover, an examination of their illustrations shows that their staining technique allowed a great spread of cholinesterase to occur away from the motor end plate into the surrounding muscle.

The results of the present researches on the gastrocnemius of the guinea-pig show that the concentration of cholinesterase at the motor end plate, as estimated by the intensity of the black copper sulphide precipitate, begins to be reduced at about the 11th day following denervation, and this reduction progresses until at the 45th day no trace of the enzyme can be detected even after one hour's incubation. The configuration of the cholinesterase was found to change following denervation. At the 15th day following nerve section the gutters became less well defined and the edges irregular. These changes were probably due to the accompanying cytological changes occurring in the muscle while undergoing atrophy (Tower, 1935). By the 22nd day the distribution of cholinesterase was found to be irregular the enzyme being highly concentrated at some points in the motor end plates while in others it could barely be detected. By the 28th day the concentration of cholinesterase was so small that it was difficult to find the myoneural junctions in the sections. The dimensions of the cholinesterase configurations in the denervated muscles were not recorded since it was considered that they would serve no useful purpose in view of the accompanying atrophy.

The fall in concentration of cholinesterase at the motor end plate in skeletal muscles following nerve section may account for the prolonged responses evoked by acetylcholine in the muscles, and also for the effects of diffusion of acetylcholine from the blood vessels to the muscle fibres in the Philipeaux-Vulpian (Philipeaux and Vulpian, 1863) and allied phenomena. The increased sensitivity of the gastrocnemius of the cat to acetylcholine (Brown, 1937; Reid, 1942) becomes apparent about 4 days after denervation and reaches a maximum at about the 6th day. Knowlton and Hines (1937) found for the rat that the increased sensitivity to acetylcholine appeared between the 2nd and 3rd days after nerve section and reached a maximum at the 7th day; thereafter the level remained constant up to 28 days when a slow decline in sensitivity set in. Although these figures cannot be correlated closely with the results reported here it must be remembered that the present work has not been carried out on a strict quantitative basis and there may well have been a considerable reduction in the activity of cholinesterase before it became apparent histochemically. Furthermore, the decline in the increased sensitivity which occurs 28 days after denervation in the rat could be due to atrophy of the muscle and a reduction or regression in the cellular receptors themselves.

The results of experiments carried out by Brücke (1937) and Couteaux and Nachmansohn (1940) on the concentration of cholinesterase in sympathetic ganglia following denervation have a bearing on the present problem. They found that following preganglionic sympathectomy the concentration of cholinesterase within the ganglia was much reduced. This reduction could account for the increased sensitivity of the ganglion cells to acetylcholine. These findings are in accord with those at the motor end plate.

Many workers have shown that denervated skeletal muscle becomes hypersensitive to many stimulating chemical agents other than acetylcholine. The hypersensitivity of denervated skeletal muscle to nicotine (Heidenhain, 1883; Langley, 1905), to potassium (Frank, Nothmann and Guttmann, 1923) and to several quaternary ammonium compounds (Dale and Gasser, 1926) cannot with

our present knowledge be explained as being due to a decrease of the cholinesterase present at the motor end plate. It is probable that denervation affects many chemical and physiological mechanisms other than the cholinesterase reaction.

SUMMARY

The effect of denervation on the histochemical appearances of cholinesterase at the motor end plate has been studied in 14 mature guinea-pigs.

The cholinesterase concentration at the motor end plate was found to begin to become reduced at about the 11th day following denervation and this reduction progressed until at the 45th day the cholinesterase was found to be absent.

The possible relationship of these findings to the increased sensitivity of denervated skeletal muscle to acetylcholine is discussed.

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EXPERIMENTAL GLOMERULO-NEPHRITIS PRODUCED IN MICE BY SUBCUTANEOUS INJECTIONS OF HEAT-KILLED *PROTEUS MIRABILIS*

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THIS investigation concerns the production of a severe glomerulo-nephritis in mice repeatedly injected with heat-killed *Proteus mirabilis* by the subcutaneous route. This procedure followed the finding (Marshall and White, unpublished experiments) that mice injected in this way in the attempt to produce amyloidosis developed gross renal changes. The localisation of antigenic material of *P. mirabilis* within the tissues of such injected mice is also described.

Disease in animals more or less resembling human glomerulo-nephritis has previously been produced repeatedly by three methods.

1. That introduced by Pearce (1903) and Masugi and Tomizuka (1931), of intravenous injection of serum from animals of another species, immunised against extracts of kidney of the test species, (*i.e.*, with anti-kidney serum) (Smadel, 1936, 1937 ; Smadel and Farr, 1936).

2. Intravenous injection of single or multiple large doses of serum proteins from normal animals of another species (Longcope, 1913 ; Rich and Gregory, 1943 ; Germuth, 1953).

3. Many attempts have been reported using bacterial agents with varying degrees of success. Bloomfield (1919) and Duval and Hibberd (1926) tried to produce glomerulo-nephritis in rabbits with streptococci injected by different routes without success. However Duval and Hibberd (1926) claimed that when living cultures of " *Streptococcus scarlatinae* " were introduced into the peritoneal cavity of immune rabbits, the toxic products resulting from lysis of the organisms caused haemorrhagic glomerulo-nephritis when injected intravenously into other rabbits. Success with a similar procedure using *Streptococcus pneumoniae* type I was also reported by Blackman, Brown and Rake (1931).

Schwentker and Comploier (1939) claimed to have made homologous kidney antigenic by mixture with staphylococcal and streptococcal toxins, but did not succeed thereby in producing nephritis. Cavelti and Cavelti (1945) reported that antibodies to rat kidney were produced by intraperitoneal injection of homologous kidney and Group A streptococci with development of glomerulo-nephritis. Their observations were not confirmed by Humphrey (1948). Glynn and Holborrow (1952) reported that a rabbit antiserum to Group A streptococci plus rat kidney digest produced glomerular lesions when given intravenously to rats.

Mixtures of serum proteins and bacteria have been reported as effective. More and Kobernick (1951) induced glomerulo-nephritis in rabbits by injections of serum protein and killed Group A streptococci intravenously or serum proteins incorporated into a Freund-type adjuvant mixture intramuscularly. In all these

experiments using bacteria in complex mixtures the rôle of the bacteria is not clear.

In some instances the intravenous injection of bacterial agents alone has resulted in the development of glomerulo-nephritis. Long and Finner (1928) produced unilateral nephritis by injection of tuberculin into the renal artery of tuberculous swine. Lukens and Longcope (1931) found focal or diffuse unilateral glomerulo-nephritis in 50 per cent of rabbits injected with heat-killed haemolytic streptococci into a renal artery. Bell and Clawson (1931) found a form of chronic diffuse glomerulo-nephritis in a monkey after intravenous injections of *Streptococcus viridans* continued over a period of four years. McLeod and Finney (1932) produced unilateral renal lesions in rabbits, simulating human glomerulo-nephritis by injection of heat-killed *Str. viridans* into a renal artery. Teilum, Engboek, Harboe and Simonsen (1951) described a glomerulo-nephritis in some rabbits injected intravenously for many months with formaldehyde-killed *Haemophilus influenzae*. The production of glomerulo-nephritis in animals by subcutaneous injection of bacteria or bacterial products has not been previously reported.

MATERIALS AND METHODS

Animals

Albino mice of a single Swiss strain were used. The mice were aged 8–12 weeks, and weighed 25–35 g. when the experiment was begun.

Vaccine

P. mirabilis was grown on nutrient agar and harvested into normal saline after 24 hr. growth at 37°. This saline suspension was then heated for 1 hr. at 56°, and after testing for the absence of viable organisms, was diluted to an opacity corresponding to 10^{10} organisms per ml.

Alcoholised P. mirabilis *suspension*

This was prepared by growing the organisms as above and suspending them evenly in the minimum of saline. About 20 times the volume of absolute ethanol was then added and the suspension heated at 45° for 30 min. The deposited organisms were subsequently suspended in saline to a density equivalent to 10^{10} organisms per ml.

Injection procedure for production of glomerulo-nephritis

Thirty-four mice were injected in the subcutaneous tissue of the trunk with 0·1 ml. of the heat-killed vaccine on 4 days of each week for a maximum period of 15 weeks. Controls consisted of 21 uninjected mice which were kept under identical conditions with the test animals and sacrificed at the same time intervals. Further controls consisted of 11 mice which received injections of 0·1 ml. of normal saline only for 4 days in each week up to a maximum period of 10 weeks.

Determination of serum agglutinins

Levels of agglutinins against H and O antigens of *P. mirabilis* were determined by the use of formolised and alcoholised suspensions of organisms grown in nutrient broth. Other determinations of agglutination titres were done with a saline suspension of organisms grown in nutrient broth and killed by exposure to a temperature of 56° for 30 min. All suspensions were diluted with saline to a density equivalent to the Standards Laboratory suspension. Of the above suspension 0·5 ml. amounts were mixed with 0·5 ml. of dilutions of serum, and the tubes incubated at $\frac{1}{2}$ immersion for 4 hr. at 56°. Serum titres were expressed as the reciprocal of the final dilution of serum producing naked-eye agglutination.

Demonstration of Antigen in Tissue Sections

Frozen sections were prepared at 4 μ thickness by the modification of the Linderstrøm-Lang and Mogensen (1938) technique, described by Coons, Leduc, and Kaplan (1951). The sections were fixed in 95 per cent ethanol for 15 min. at 37°.

P. mirabilis antigens in the frozen tissue sections were demonstrated by the technique of Coons and Kaplan (1950). Briefly, the procedure involved the precipitation of fluorescein-tagged *P. mirabilis* antibody, when a solution of the latter was layered over the tissue section and allowed to react for 30 min. The excess non-precipitated antibody was removed by a brief wash (10 min.) in buffered saline (pH 7·0). The preparation was mounted in buffered saline (pH 7·0) and sealed with a Vaseline-paraffin wax mixture.

The preparation of the fluorescein-labelled antibody was as described by Coons and Kaplan (1950). The preparation of the antiserum in the rabbit is described by White (1954), together with details of the staining procedure. All conjugates were absorbed before use with acetone-dried mouse liver powder, prepared as described by Coons, Leduc and Connolly (1955), for the avoidance of non-specific staining by the crude conjugate. The equipment for fluorescence microscopy was that described by Coons, Creech, Jones and Berliner (1942), modified by the use of a Zeiss cardioid dark-field condenser (Coffin, Coons and Cabasso, 1953).

Antigen was demonstrated by the fluorescence microscope as bright apple-green areas. The frozen sections of mouse liver, spleen, and kidney have no visible structures with this colour of primary fluorescence, although blue, red and yellow are present. Crude fluorescein-labelled globulins make many structures of normal tissues fluorescent. Most of this is easily avoided by previous absorption of conjugates with acetone-dried mouse liver powder. Even after this precaution the cytoplasmic granules of eosinophile leucocytes may give rise to confusion but may be recognised, since the fluorescence is more orange than green and the cytoplasmic outlines surrounding the usually bilobed nuclei are characteristic.

No non-specific fluorescence has been found in renal glomeruli treated with fluorescein-globulin conjugates. However, certain tubules have occasionally been found to give non-specific fluorescence, standing out in sharp contrast with adjacent non-fluorescent ones. This appearance could be completely avoided by absorption of the fluorescein conjugates with certain batches of mouse liver powder but not by others, even though the absorption procedure was repeated many times.

Areas of fluorescence in kidney glomeruli were further investigated by the following tests to establish that the observed staining was immunologically specific.

Exposure to heterologous conjugates.—In order to determine whether a given area of fluorescence was due to a non-specific precipitation of fluorescein-labelled globulins control sections were exposed to conjugates prepared from rabbit antisera to antigens other than *P. mirabilis*. Areas of fluorescence were only accepted as specific when these procedures were negative.

Inhibition of staining with unconjugated homologous antiserum.—In order to perform such inhibition successfully it was necessary to expose sections to unconjugated high titre rabbit antiserum for *P. mirabilis* for a long period. Thus frozen sections, after fixation with 95 per cent ethanol for 15 min., were dried at 37° for 30 min., rehydrated with normal saline and then exposed to a 1/40 dilution of unconjugated *P. mirabilis* antiserum for 2 hr. They were then washed in saline for 4 min. and stained with the *P. mirabilis* fluorescein conjugate diluted 1/10 for 5 min., washed in saline for 10 min., mounted and examined. A control adjacent section was treated under the same conditions except that normal rabbit serum was substituted for the unconjugated antiserum. By this procedure complete inhibition of staining could not be achieved, but such sections were obviously less brightly stained than the controls.

Inhibition of staining by absorption of fluorescein conjugate with suspensions of P. mirabilis.—To 0·1 ml. of *P. mirabilis* fluorescein conjugate was added 0·9 ml. normal saline containing 4×10^{10} heat-killed or alcoholised *P. mirabilis*, and the mixture allowed to stand for 1½ hr. at room temperature with repeated shakings. The suspensions were then centrifuged at 6000 r.p.m. for 1 hr. and the supernatant recovered.

Staining with conjugates absorbed in the above manner was always done in conjunction with controls which were unabsorbed conjugates diluted 1/10, which were allowed to react for the same time as the absorbed reagents. Fluorescent material was accepted as antigens

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of *P. mirabilis* only after the demonstration that the above absorption procedures resulted in complete absence of fluorescence in areas which were still fluorescent in the control sections.

RESULTS

Findings in Mice Injected with P. mirabilis *for the Production of Glomerulo-nephritis*

Diffuse glomerulo-nephritis was found in 16 out of 32 mice. These mice had been killed at increasing intervals and thus had received repeated injections of *P. mirabilis* for periods varying from 1–15 weeks. They included 5 mice which died. No glomerulo-nephritis was present in any of the 21 uninjected control animals or the 11 control mice injected with saline. Renal infection with the protozoal parasite *Klosiella muris* was found in 11 mice injected with *P. mirabilis* and 9 controls. The presence of *Klosiella* in renal tubules was associated with focal interstitial lymphocytic infiltration and slight tubular dilatation and atrophy. Diffuse glomerular changes, characteristic of the glomerulo-nephritis found only in mice injected with *P. mirabilis* were not correlated with *Klosiella* infection. However, those animals in which *Klosiella* were found are excluded from the remainder of this report. Of the remaining 21 mice injected with *P. mirabilis* and examined after 1 to 11 weeks, glomerulo-nephritis was present in 10, no glomerulo-nephritis occurring in 12 uninjected or 11 saline-injected controls (Table I). The earliest example of nephritis was seen after 2 weeks. This mouse had received an estimated 8×10^9 organisms by subcutaneous injection, equivalent to a total of 5·4 mg. dried bacterial substance. Glomerulo-nephritis was found in 10 out of 19 mice examined after 2 or more weeks. The increasing incidence of nephritis as the experiment continued suggests that the time of onset of nephritis varied widely.

TABLE I.—*Incidence of Glomerulo-nephritis in Mice Injected with* P. mirabilis

Injection period.	No nephritis.	Acute nephritis.	Subacute nephritis
1 week	2	—	—
2 weeks	1	1	—
3 ,,	1	1	—
4 ,,	2	—	—
5 ,,	1	—	—
6 ,,	—	1	1
7 ,,	1	—	1
8 ,,	2	—	—
9 ,,	—	1	—
10 ,,	1	1	1
11 ,,	—	2	—
Total	11	7	3
Controls	23	—	—

The renal changes were those of acute or subacute diffuse glomerulo-nephritis. Acute nephritis was found in 7 mice after injection periods that ranged from 2 to 11 weeks. Subacute nephritis was seen in 3 animals, the earliest being found after 6 weeks.

The mice injected with *P. mirabilis* showed, on average, a very slight weight increase (0·1 g. per week) throughout the experiment. There was considerable departure from the average weight change in individual mice, but no significant difference between the group of injected mice with no renal lesions, and those showing nephritis at post-mortem. The uninjected controls showed a steady weight increase (average 1·1 g. per week). Anasarca was observed in only one mouse, in which subacute nephritis was present after an injection period of 10 weeks. This animal showed subcutaneous oedema, especially over the anterior thorax.

Five mice injected with *P. mirabilis*, but none of the controls, died during the experiment. Three of the mice that died did not show nephritis at post-mortem. Two of these died within one week of starting injections, and one had acute bronchopneumonia. The mice with nephritis that died both showed subacute nephritis, after injection periods of 6 to 7 weeks. Only one mouse of the series appeared severely ill, being unresponsive on one day and breathing rapidly. It was killed the same day, and at post-mortem showed subacute nephritis, with anasarca and a blood urea of 360 mg. per 100 ml.

Microscopic haematuria was seen in urine from 2 mice out of 6 examined, after injection periods of 10 weeks. Urine specimens from both animals had previously been free of cells. The kidneys of both mice, when killed after 11 weeks, showed acute nephritis with tubular haemorrhage.

Urea was estimated in blood collected at the time of death from most of the mice. The range in all the controls, and in injected mice without nephritis, was 30–42 mg. per 100 ml. Slightly higher values were found in 3 mice with acute nephritis (each 60 mg.) and a marked increase (360 mg.) was present in 1 mouse with subacute nephritis.

Serum from blood collected at the time of death was used to determine agglutination titres (Table II). High levels of circulating antibodies against both somatic and flagellar antigens of *P. mirabilis* were found in the test mice but not in the controls; there was no significant difference between antibody levels in nephritic and non-nephritic mice of this group.

TABLE II.—*Mouse Serum Agglutination Titres, with Saline (heat-killed), Alcoholised (O) and Formolised (H) Suspensions of* P. mirabilis

Injection period.	No nephritis.	Nephritis.		
	Saline.	Saline.	O.	H.
1 week	60	—	—	—
2 weeks	—	30	—	—
3 ,,	1920	—	—	—
4 ,,	240	—	—	—
	3840	—	—	—
5 ,,	1920	—	—	—
6 ,,	—	1920	—	—
7 ,,	960	—	—	—
8 ,,	120	—	—	—
9 ,,	—	3840	—	—
10 ,,	7680	240	3840	1920
11 ,,	—	—	1920	480
	—	—	3840	1920

Controls (4) negative at 1/30.

Macroscopic examination

At post-mortem the kidneys in acute nephritis were slightly enlarged with smooth surfaces of normal colour. In subacute nephritis the kidneys were considerably enlarged. The kidney-body weight ratio was about twice that of controls. The renal surfaces were pale and in one mouse finely granular with numerous red points. There was no definite evidence of cardiac hypertrophy at any stage. It is therefore unlikely that any persistent elevation of blood pressure was present. Blood-pressure measurements were not undertaken. The spleens of mice injected with *P. mirabilis* with acute nephritis or without nephritis, were enlarged. In all animals of both groups, the spleen/body weight ratio was over twice that of controls. The spleen was enlarged in only 1 of the 3 mice with subacute nephritis. In all the mice injected with *P. mirabilis*, indurated subcutaneous swellings were found at the sites of recent injection in the back or abdomen. The skin over these swellings was intact. No other gross lesions were observed.

Microscopic examination

Acute nephritis.—Sections of kidney in the 7 mice with acute nephritis showed diffuse glomerular enlargement and increased cellularity, due to proliferation of cells of the glomerular tuft (Fig. 1 and 2). In many instances measurements of individual glomeruli in serial sections showed that all the glomeruli were enlarged. Equatorial glomerular diameters ranged from 102–120 μ up to 130–160 μ (normal 82–98 μ). Leucocytic infiltration of the glomeruli was slight or absent. There was marked glomerular capillary ischaemia, apparently due to obliteration of capillary lumina by cellular proliferation in the tufts. Formation of hyaline material, with fibrinoid staining in peripheral capillary loops of glomerular tufts was occasionally seen (Fig. 3). This was frequently associated with adhesion to the adjacent epithelium of Bowman's capsule, and with focal proliferation and thickening of the capsular epithelium. No thickening of glomerular capillary basement membranes was apparent in periodic acid Schiff (P.A.S.) or azan-stained sections.

Tubular haemorrhage was found in 3 mice with acute nephritis (Fig. 4). Small hyaline casts occupied occasional tubules. Hyaline droplets stained with P.A.S. affected the epithelium of occasional proximal convoluted tubules (Fig. 5). There was no associated fatty degeneration. There was no leucocytic infiltration in interstitial tissue. Apart from ischaemia and fibrinoid formation in glomerular capillaries, there were no vascular lesions.

Subacute nephritis.—The renal changes of acute nephritis were also found in the three mice with subacute nephritis. In addition there was focal hyaline necrosis in the glomerular tufts and extensive capsular adhesions. Many of the glomeruli showed large capsular crescents, consisting of epithelial cells of Bowman's capsule and fibroblasts with collagen fibres (Fig. 6). Hyaline material was seen in capsular spaces around some tufts and hyaline casts were very numerous in the tubules. The lumina of many tubules were distended and the tubular epithelium flattened by the casts they contained. There was slight to moderate focal infiltration of the interstitial tissue by lymphocytes, histiocytes and plasma cells, and slight diffuse interstitial fibrosis which was rather more accentuated about the glomeruli. No changes were found in the glomerular arterioles or in the other arteries of the kidneys.

The heart showed no abnormality. There was no hypertrophy of myocardial fibres, and no evidence of myocarditis. The spleens of mice injected with *P. mirabilis*, with or without nephritis, showed reticulum cell hyperplasia and moderate infiltration of the pulp by polymorphonuclear leucocytes. These changes were absent from one mouse with subacute nephritis and an unenlarged spleen in which plasma cell infiltration alone was seen in the pulp. No granulomata were found in the spleen or liver, the latter appearing normal. No lesions suggesting polyarteritis nodosa, or any other vascular changes, were found. Except for suppurative bronchopneumonia in one mouse, which died in the first week of the experiment, and pulmonary oedema with recent alveolar haemorrhage in one mouse with subacute nephritis which died after 7 weeks, there were no pulmonary lesions.

Additional experiment

In the foregoing experiment a single strain of *P. mirabilis* from a human source was used. Twelve further mice were injected under the same conditions with suspensions of *P. mirabilis* cultivated from another patient. The animals were examined at intervals up to a maximum of 12 weeks. Nephritis was present in only one of these mice, killed after 3 weeks. This mouse showed slight subcutaneous oedema and ascites, and a weight increase to 36 g. from 28·5 g. at the start of the experiment. The blood urea was 198 mg. per 100 ml. The kidneys showed acute diffuse glomerulo-nephritis, with the same features as in the previous experiment. Serum agglutinating titres were 120 (O) and 1920 (H). In two non-nephritic mice serum antibody levels were 7680 (O) and 1920 (H), after 4 weeks' injections ; 1920 (O) and 3840 (H) after 9 weeks.

The Distribution and Subsequent Fate of P. mirabilis *in Mice*

The following is a brief account of experiments designed to trace the fate of antigenic material from *P. mirabilis* within mouse tissues by fluorescence microscopy. Frozen sections obtained at varying time intervals after injection were exposed to fluorescein-labelled *P. mirabilis* antibody. The presence of antigenic material was denoted by apple-green fluorescent areas in such sections. Before, finally accepting such appearances controls for non-specific deposition of fluorescein conjugates were applied as described above.

Intravenous injection

When heat-killed *P. mirabilis* were injected intravenously sections of the liver and spleen from animals killed at short intervals thereafter demonstrated the rapidity of the process of phagocytosis by the sinus-lining cells of the reticulo-endothelial system. At 15 min. after injection large numbers of *P. mirabilis* were seen within Kupffer cells (Fig. 9). Already, too, digestion of the bacilli had occurred, so that irregular remnants of bacilli and amorphous fluorescent material representing diffusible antigens could be seen (Fig. 9). The process of digestion was such that at 18 hr. after injection all the antigen within the Kupffer cells was in the form of fine dust-like granules or diffusely fluorescent segments of cytoplasm.

Moreover, such antigenic débris appeared to persist intracellularly and was still detectable at 3 weeks after injection.

Subcutaneous injection

Following a single subcutaneous dosé of 4×10^9 heat-killed *P. mirabilis*, sections of the spleen, liver and kidney from animals sacrificed 24 hr. after injection failed to show any content of antigen, with the exception that polymorphs with a single contained bacillus could sometimes be seen in a hepatic sinus. At 3 days after injection definite amounts of antigen could be detected in sinus-lining macrophages of the liver and in the glomeruli of the kidney. The amounts of such antigen in sections of liver and kidney increased with longer intervals up to a maximum at 7 days. Fig. 10 shows amorphous antigen in the Kupffer cells at 7 days after injection. Small amounts of such material could still be observed in liver and kidney at 3 weeks after injection. Fig. 11 shows antigen in the kidney glomerulus 18 days after injection. In certain cases it was possible to detect antigen in kidney glomeruli at 3 weeks when none could be detected in the liver. The antigen appeared to be at the surface of and within the glomerular epithelial cells. Antigen persisted locally at the site of injection. At 3 weeks no intact bacilli could be seen. The cytoplasm of microphages and macrophages contained bright granules and diffuse areas of antigen. The main bulk of antigen was intracellular.

Multiple subcutaneous injections

When mice were injected subcutaneously with repeated doses of heat-killed organisms at time intervals as described above for the induction of glomerulo-nephritis, antigen in large amounts accumulated in the glomeruli of the kidney, and throughout the cells of the reticulo-endothelial system in the liver and spleen (Fig. 13). All the glomeruli of such animals contained antigen. No fluorescence indicative of the presence of antigen occurred in renal structures other than glomeruli in non-nephritic mice or mice with acute glomerulo-nephritis. The intra-glomerular localisation of antigen was studied with the help of photographs of the same glomerulus in the next adjacent serial section, fixed in formalin and stained with haematoxylin and eosin (Fig. 7 and 8). Antigen was seen in the glomeruli as bright patches, often enclosing dark areas corresponding in size and shape to nuclei. These dark areas were not thought to represent capillary lumina, because they were neither much elongated nor crescent-shaped, and because they were just as numerous in ischaemic glomeruli in which the capillary lumina appeared to be obliterated. The appearance did not suggest that the antigen was deposited on basement membranes and in this connection it is instructive to compare the appearances of fluorescence which is confined to basement membranes (Hill and Cruickshank, 1953). Rather the appearances were interpreted as deposition of antigen within the cytoplasm of glomerular cells. Except for alteration in the configuration of the glomeruli due to enlargement and increased cellularity there was no difference in amount or site of antigen in kidneys showing acute nephritis or kidneys of non-nephritic injected mice. In the sections of kidneys with subacute nephritis, antigen was particularly abundant and was no longer confined to the glomerular tufts, but appeared in the capsular epithelium and crescents (Fig. 12). Antigen was also present at this stage in some tubular hyaline casts as a patchy fluorescence.

DISCUSSION

The glomerulo-nephritis produced in mice by subcutaneous injections of heat-killed *P. mirabilis* resembles type 1 glomerulo-nephritis in man (Ellis, 1942) in the following features.

(1) Bacteria, or their antigens, are an important initiating factor.

(2) Haematuria, and the histological observation of free red corpuscles in glomerular capsules and in the lumina of tubules.

(3) Progression of the nephritis to a " subacute " stage accompanied by renal failure.

(4) Histological features recorded above in both the acute and subacute stages, in particular, the proliferative changes in the glomeruli, fibrinoid necrosis in glomerular tufts, hyaline droplet degeneration in tubular epithelium, formation of large capsular epithelial crescents, extensive capsular adhesion, and pericapsular fibrosis.

The experimental nephritis also differs from Ellis' type 2 nephritis in man.

(1) In the absence of generalised oedema as a predominant symptom.

(2) Absence of secondary infection in the mice that died.

(3) Absence of lipid degeneration in tubular epithelium.

(4) Absence of thickening of glomerular capillary basement membranes.

It appears that this form of experimental nephritis resembles human type 1 nephritis more closely than does anti-kidney serum nephritis. Thus Heyman and Lund (1948) gave the name of lipaemic nephrosis to the disease in rats produced by rabbit anti-rat kidney serum. They emphasised the marked oedema and absence of haematuria. The kidneys showed tubular degeneration, but glomerular changes were slight. Smadel (1937) described this form of glomerulo-nephritis as characterised by swelling of the intercapillary substance of the glomerular tuft. These are characteristic features of human type 2 nephritis.

The site of localisation of *P. mirabilis* antigens in glomerular cells in the injected mice may be compared with the reported localisation of agents concerned in the production of anti-kidney serum nephritis. Antibody in nephrotoxic serum has been shown to be directed against a constituent of basement membranes, by high antigenic activity of basement membranes, separated from isolated glomeruli by sonic vibration (Greenspon and Krakower, 1950), and by application of fluorescein-conjugated globulin fractions of antisera to sections of normal rat kidney (Hill and Cruickshank, 1953). It was shown that the globulin from anti-whole kidney serum reacted with basement membranes in glomeruli and convoluted tubules and with the cytoplasm of tubular epithelium. Antibody globulin against lung tissue and glomeruli reacted with renal basement membrane but not with tubular epithelium.

Localisation of antigens concerned in forms of experimental nephritis other than that produced by anti-kidney serum has received less attention. Mellors, Arias, Siegal and Pressman (1955) used fluorescein-labelled chick anti-rabbit globulin to demonstrate localisation of rabbit globulin in tissue sections. The glomeruli of rabbits rendered nephritic by injection of bovine gamma globulin showed an increased content of rabbit globulin as compared with controls. Such locally concentrated globulin may represent antibody to glomerular-localised

foreign protein. The renal localisation of bacterial antigens in nephritis produced by injection of bacterial agents has not been previously reported.

Antigenic material from a single subcutaneous injection of *P. mirabilis* accumulates within the cells of the kidney glomeruli and persists there at least for several weeks. This suggests the hypothesis that the nephritic process results as an immunological reaction to the presence of such " foreign " antigen within kidney glomeruli. In this instance the nephritic process could be regarded as analogous to the tissue reaction which results in the rejection of a heterograft (Medawar, 1944), or the delayed-type cellular reaction. Alternatively nephritis could be regarded as the result of the interaction between humoral antibody produced elsewhere and antigen localised in kidney glomeruli.

The differences in histological appearance between the nephritis produced by *P. mirabilis* and anti-kidney serum nephritis could on either hypothesis be dependent on the different site of localisation of antigenic material. Comparisons are difficult since factors such as dosage of antigen and species of experimental animal may affect the histological picture (Ehrich, 1953).

EXPLANATION OF PLATES

Except where it is stated otherwise, photomicrographs refer to tissue sections from mice injected subcutaneously with heat-killed *P. mirabilis* on 4 days of each week.

Fig. 8–13 are photomicrographs of frozen sections taken under the fluorescence microscope. The light areas represent the yellow-green light from fluorescein and indicate sites of *P. mirabilis* antigen in the tissue sections. In some cases the normal tissue is faintly outlined by the less intense blue-grey autofluorescence.

FIG. 1.—Normal glomerulus of uninjected mouse. Paraffin section. H. and E. × 450.

FIG. 2.—Acute glomerulo-nephritis. Enlargement of glomerular tuft by cellular proliferation. Mouse injected for 3 weeks. Paraffin section. H. and E. × 450.

FIG. 3.—Acute glomerulo-nephritis. Hyaline formation in glomerular capillary loops with capsular adhesion and early capsular epithelial proliferation. Mouse injected for 9 weeks. Paraffin section. H. and E. × 450.

FIG. 4.—Acute glomerulo-nephritis. Haemorrhage in glomerular capsular space and tubules. Mouse injected for 9 weeks. Paraffin section. H. and E. × 330.

FIG. 5.—Acute glomerulo-nephritis. Hyaline droplet formation in epithelium of proximal convoluted tubule. Hyaline casts in distal convoluted tubules. Mouse injected for 2 weeks. Paraffin section. P.A.S. × 330.

FIG. 6.—Subacute glomerulo-nephritis. Capsular epithelial and fibrous crescents. Focal hyaline necrosis in glomerular tufts with capsular adhesions. Mouse injected for 6 weeks. Paraffin section. van Giesen. × 135.

FIG. 7.—Acute glomerulo-nephritis. Glomerulus of mouse injected for 6 weeks. Frozen section 4 μ thick. H. and E. × 600.

FIG. 8.—The same glomerulus as in Fig. 7, in adjacent serial section 4 μ thick. *P. mirabilis* antigen in glomerular cells. Fluorescence micrograph. × 600.

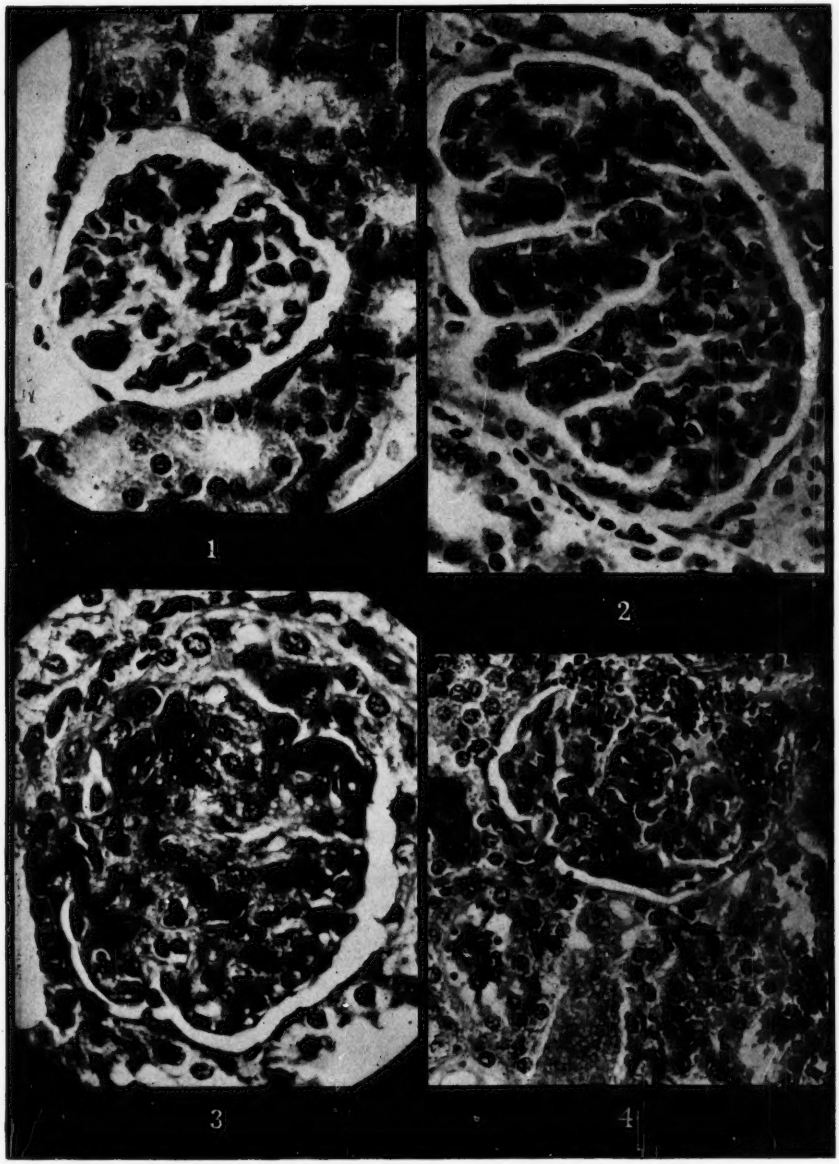
FIG. 9.—Mouse liver. Frozen section treated to reveal *P. mirabilis* antigens. Intact organisms, disintegration forms and amorphous areas of antigen within the cytoplasm of Kupffer cells 15 minutes after intravenous injection. Fluorescence micrograph. × 640.

FIG. 10.—Fine granular and amorphous antigen of *P. mirabilis* within the cytoplasm of Kupffer cells, 7 days after a single subcutaneous injection. Fluorescence micrograph. × 660.

FIG. 11.—Antigen in renal glomerulus 18 days following a single subcutaneous injection. Fluorescence micrograph. × 500.

FIG. 12.—Renal glomerulus from mouse with subacute stage of glomerulo-nephritis. Antigen in glomerulus and (above and to left) in cells of a capsular crescent. Fluorescence micrograph. × 320.

FIG. 13.—Spleen. *P. mirabilis* antigen in cells throughout medulla. The lymphoid nodules are devoid of antigen. Fluorescence micrograph. × 77.



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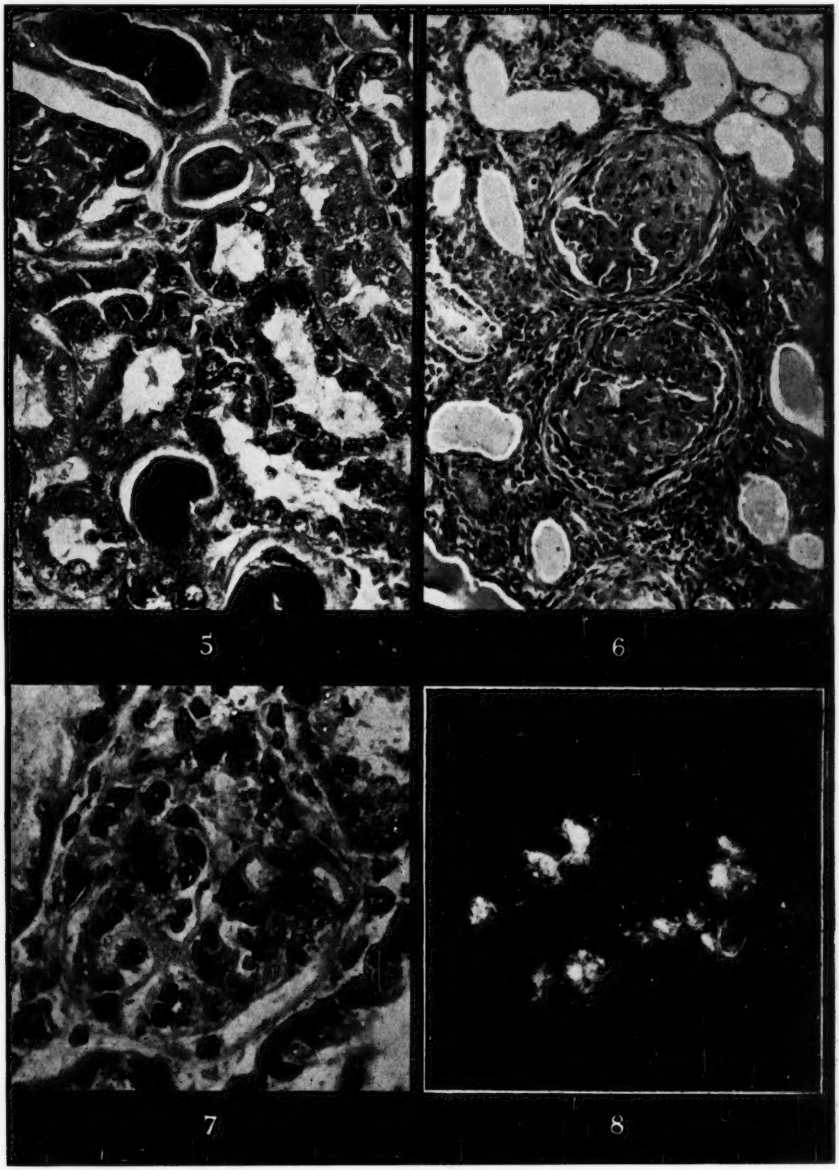

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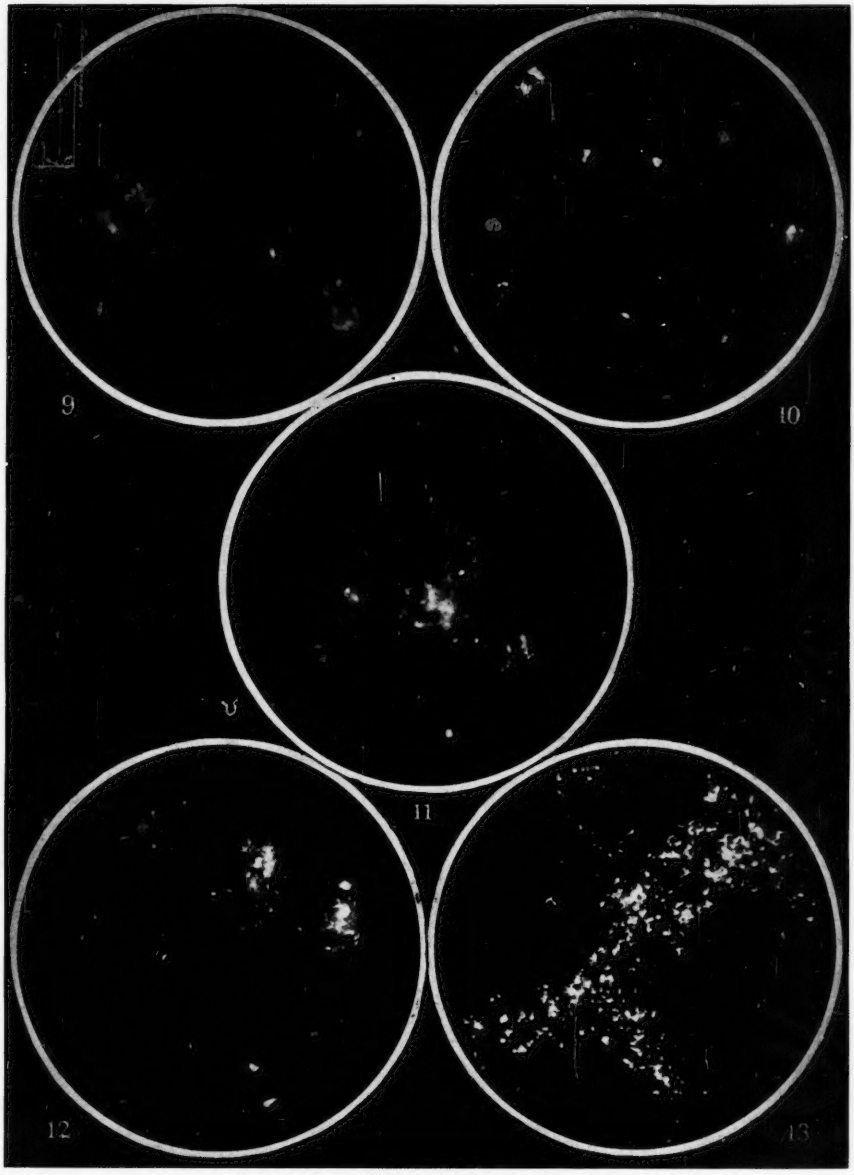

Wood and White.

It is realised, of course, that the nephritis produced in these animals with injections of *P. mirabilis* may be quite unrelated to the antigenic activity of the material which localises in the kidney glomeruli.

The results of the use of a different strain of *P. mirabilis* in which nephritis was produced in only one animal, indicate that different strains may vary in capacity to cause this lesion in mice.

SUMMARY

Acute or subacute diffuse glomerulo-nephritis was produced in about 50 per cent of mice by repeated subcutaneous injections of heat-killed *P. mirabilis*, continued for 2 to 11 weeks. Using fluorescein-labelled antibody, antigenic material from *P. mirabilis* was found to be localised in the glomerular cells. After a single subcutaneous dose antigen was shown to persist for at least 3 weeks in glomeruli.

It is suggested that the localisation of bacterial antigen in glomerular cells may be causally related to the production of nephritis and that this localisation site is related to the type of glomerulo-nephritis produced in mice, which resembled Ellis' type 1 nephritis in man.

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MANY authors maintain that the members of a cytological family whose mature member is the plasma cell are responsible for formation of antibody globulin (Bjørneboe and Gormsen, 1943; Fagraeus, 1948; Marshall and White, 1950; Ringertz and Adamson, 1950; Keuning and van der Slikke, 1950; Coons, Leduc and Connolly, 1955). When an antigenic stimulus is administered intravenously the multiplication and differentiation of these cells has been found to occur mainly in the spleen, liver and lung. When the antigen is distributed by lymphatics from a subcutaneous focus such cells appear in the regional lymph nodes. In the latter instance, and especially when the antigen is mixed with "adjuvants", local granuloma formation takes place at the site of injection. Under certain circumstances plasma cells compose a fair proportion of the cells of such granulomata. When the adjuvant is alum or aluminium phosphate considerable numbers of plasma cells are present at the site of injection and such cells have been regarded as a source of antibody (White, Coons and Connolly, 1955). On the other hand, local formation of antibody in granulomata produced by alum adjuvant has been regarded by Hartley (1940) as evidence that the macrophages function in this respect. Similarly, the local cellular response following the use of Freund-type adjuvants (Freund and McDermott, 1942), in which the antigen is administered in a water-in-oil emulsion with added *Mycobacterium tuberculosis*, has given rise to the view that the cells assembled near the site of injection are responsible for antibody production (Freund, 1951). The main cell component of such granulomata is the macrophage.

Analysis of such problems has been attempted by the extraction of antibody from the local site at varying time intervals after injection of antigen. Westwater (1940*b*) showed by such studies that complement-fixing antibody to tubercle bacilli appeared earlier in the lesion at the site of injection than in the blood. This method only proves *localisation* of antibody; that *production* of antibody occurs at the site of its localisation requires further support.

Further evidence for determining the source of such antibody was provided by the method for the cytological localisation of antibody (Coons *et al.*, 1955). Applied to the guinea-pig (White *et al.*, 1955), this method showed that the macrophages of the local granuloma were devoid of antibody. On the other hand, large numbers of antibody-containing plasma cells were present in the lymph nodes remote from the site of injection, and in the spleen.

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The present investigation was undertaken to correlate these findings with the ability of the isolated tissues to synthesise specific antibody when incubated *in vitro*, using the method of Askonas and Humphrey (1955). Data are also provided concerning the synthesis of γ-globulin under the same experimental conditions. Preliminary findings of this study have been reported (Askonas and White, 1955).

MATERIALS AND METHODS

Antigen and mode of administration

The antigen used throughout this study was crystalline egg albumin (Armour and Co., Ltd.) in doses of 5 mg. per injection. This was given in 0·2 ml. of a water-in-oil emulsion into the subcutaneous tissue of the footpad. This site was chosen so that draining lymph nodes in the popliteal fossa and those of the flank could be conveniently used. The injection mixture was composed of 1 vol. of a saline solution of antigen, 1 vol. of Arlacel A (batch 3375, Atlas Powder Company, Wilmington, Delaware) and 3 vol. Bayol F (Esso Standard Oil Company). Tubercle bacillary wax (lipopolysaccharide or wax D fraction of the human strain " Canetti "), kindly supplied by Dr. E. Lederer of the Institut de Biologie Physico-chimique, Paris, was dissolved in the Bayol F before emulsification to give a dose per injection of 1 mg.

Animals

Guinea-pigs of weight 350–500 g. were used. They were fed on a pellet diet with supplementary cabbage *ad lib.*

Preparation of tissues

Animals were killed 3 weeks after injection by inhalation of chloroform. Immediately after death the blood was removed by heart puncture. The granuloma in the injected footpad was dissected free from the underlying metatarsal bones and the overlying skin. Samples from this and other organs were immediately weighed, and preserved for histological study by placing against the wall of thin glass test-tubes and quickly freezing in alcohol-CO_2 mixtures at − 70°. They were stored at − 20° until frozen sections could be prepared.

The remainder of the tissues was sliced with an automatic chopper (McIlwain and Buddle, 1953) and with scissors by hand and then incubated to estimate their ability to synthesise antibody *in vitro*. Known weights of sliced tissue (0·1–1·0 g. depending on the level of activity) were incubated in siliconed flasks at 37° in an isotonic salt mixture at pH 7·4 (Raker, Taylor, Weller and Hastings, 1950) containing 0·3 per cent glucose in a total vol. of 4 ml. Each flask contained 1·7 mg. of a complete amino acid mixture (acid-hydrolysed rabbit plasma protein plus tryptophan), 1 ml. of the animal's own serum and 2–3 μc of uniformly labelled ^{14}C-glycine (0·05 μc per μg.) Carbon dioxide (5 per cent) in oxygen was passed continuously through the flask and mechanical shaking was maintained throughout.

The reaction was stopped after 3–4 hr. by adding sodium ethylmercury-thiosalicylate (Merthiolate : Eli Lilly and Co.) to a final concentration of 1/10,000 ; antiserum was added so that each flask contained at least 5 mg. of antibody, and in order to dilute the radioactivity of the glycine in the system, inactive glycine was added to produce a 1/1000 dilution. The incubation mixture was frozen by immersing the flasks in a solid CO_2-ethanol mixture. After thawing the cells were broken in a glass homogeniser of the Potter-Elvehjem type and the cellular particles centrifuged down at 18,000 g. at 1°. The supernatant contained the anti-ovalbumin and γ-globulin fractions.

Isolation of specific antibody precipitate

Antibody was precipitated from the supernatant by addition of the calculated amount of ovalbumin, assuming an optimal precipitating ratio for ovalbumin to anti-ovalbumin of approximately 1 : 10. This ratio had been determined by the construction of precipitation curves relating weight of precipitates to antigen concentration for several antisera from guinea-pigs immunised with ovalbumin in adjuvant mixtures similar to those used in these experiments. Precipitation was allowed to continue during 15 min. at 37° and overnight at 0–4°. Precipitates were washed three times with 1 per cent glycine-saline, once with distilled water, once with hot ethanol and dried with ether.

Preparation of γ-globulin fraction

After removal of the specific antibody precipitate, a γ-globulin fraction was prepared by the method of Kekwick (1940) for human γ-globulin. The protein was precipitated first with 18 per cent w/v of sodium sulphate. It was then reprecipitated twice with 15 and 12 per cent of sodium sulphate. A small volume of saline was added and the protein soluble in saline was dialysed against distilled water and freeze-dried. This represented the γ-globulin fraction. This fraction is not homogeneous in guinea-pigs, but contains mostly the components of serum which move slowest during electrophoresis at pH 8·4 in veronal buffer, $I = 0{\cdot}2$.

Determinations of radioactivity

Dried protein was plated at infinite thickness on 0·3 or 1 sq. cm. disks and radioactivity determined by counting in an automatic recording Geiger counter with a thin-end window to an accuracy of at least ± 5 per cent.

Morphological techniques

Frozen sections at 4μ thickness were prepared from the unfixed tissue samples by the techniques of Linderstrøm-Lang and Mogensen (1938) as modified by Coons, Leduc and Kaplan (1951). The fixation and histochemical demonstration of anti-ovalbumin followed the method of Coons, Leduc and Connolly (1953, 1955) as applied to this antibody (White *et al.*, 1955). Fluorescence microscopy was carried out using equipment as described by Coons, Creech, Jones and Berliner (1942) including the modification of a dark field condenser (Zeiss cardioid) (Coffin, Coons and Cabasso, 1953). Anti-ovalbumin was seen in sections viewed with the fluorescence microscope as bright apple-green areas. A control section for the detection of ovalbumin antigen, which will also reveal any non-specific staining due to the use of the fluorescein-antibody conjugate, was always set up at the same time as that for the detection of specific antibody. A description of this control and of such non-specific staining in the lymphoid tissues of the guinea-pig is given in White *et al.*, 1955.

Counts of antibody-containing cells in tissue sections

Sections stained for anti-ovalbumin were scanned under the fluorescence microscope using a 3·65 mm. oil-immersion objective and a × 4 ocular, into which was incorporated a divided square field of 6 mm. side. The total area of section seen within the boundaries of this square was 0·0324 sq. mm. The cells within an area of 30–60 squares were counted and results were expressed as counts per unit square. Since the distribution of such antibody-containing cells is not uniform throughout a lymph node, such counts only gave a very rough indication of relative cell densities. Moreover, counts made in that portion of a bisected node which is sectioned and studied morphologically can give only a vague idea of the numbers of cells in the other portion which was incubated *in vitro*.

RESULTS

Histological Findings

All animals were killed 21 days after the single injection of ovalbumin-adjuvant mixture into the left foot-pad. The following is a simplified summary of the histological findings at autopsy. For fuller details reference should be made to two publications (White *et al.*, 1955; Suter and White, 1954) dealing with identical findings with similar adjuvant mixtures. The cytological terminology has been defined previously (Marshall and White, 1950).

Granuloma at site of injection.—The foot was greatly swollen due to the presence of firm, yellowish-grey tissue, mainly in the plantar region. This lesion was a mixed-cell granuloma. The predominating cells were macrophages, often modified to epithelioid cells and giant cells, forming a solid proliferation around oil vacuoles. Lesser numbers of polymorphs and eosinophiles were present. Plasma cells were rare.

Sections which were treated to reveal antibody to ovalbumin showed that the main mass of cells—the macrophage elements—were devoid of fluorescence. Antibody-containing cells were very scanty and limited to groups of 2–3 cells in the adventitia of small vessels (Plate I). Adjacent sections stained with Giemsa revealed these as mature and immature plasma cells. The presence of large amounts of extracellular antibody was indicated by brilliant fluorescence of the surface of collagen fibres in the area.

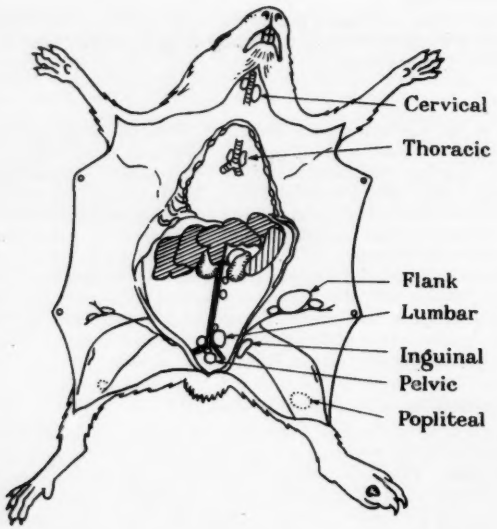


FIG. 1.—Drawing of a guinea-pig injected with the ovalbumin-adjuvant mixture in the left foot-pad, showing the position of the lymph nodes which were studied.

Lymph nodes.—The regional lymph nodes of the homolateral popliteal fossa were enlarged to 3–5 times normal mass (Fig. 1). Their normal structure was almost totally replaced by granulomatous tissue consisting of macrophages. Sections treated to demonstrate the presence of antibody showed a few antibody-containing

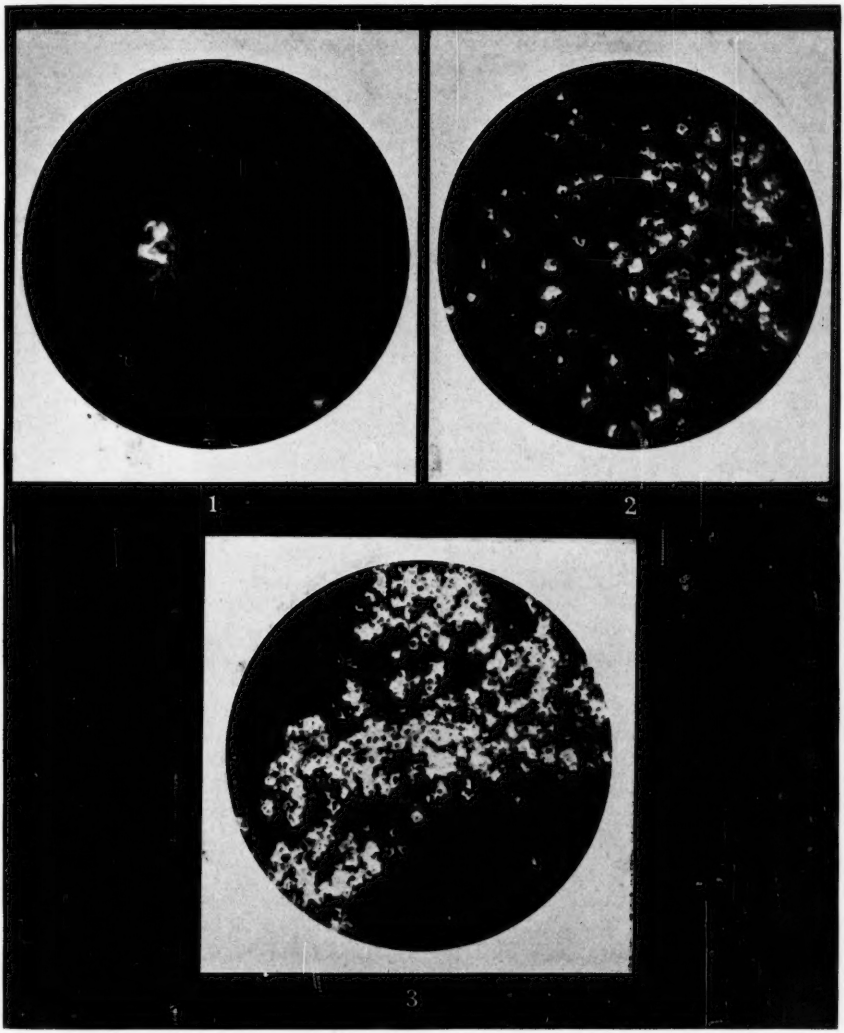
EXPLANATION OF PLATES

Plates I–III are fluorescence micrographs of frozen sections of guinea-pig tissues which were treated to reveal the distribution of anti-ovalbumin. The light areas represent the deposited fluorescein-labelled anti-ovalbumin which appeared bright apple-green in the original preparations. The primary blue fluorescence of the tissues gives a dim outline of other structures.

PLATE I.—Three antibody-containing plasma cells in the granuloma at the site of injection of ovalbumin-adjuvant mixture in the foot-pad of the guinea pig. The antibody outlines the cytoplasm of these cells. ×840.

PLATE II.—Homolateral flank lymph node. The bright cytoplasmic outlines of antibody-containing plasma cells are seen scattered over the section. × 190.

PLATE III.—Contralateral lymph node. Dense collections of antibody-containing plasma cells throughout a medullary cord between the edges of two lymphoid nodules (above left and below right) which are devoid of fluorescence. × 190.



Askonas and White.

cells, identifiable as immature or mature plasma cells by reference to adjacent Giemsa-stained sections.

Strikingly enlarged to 8–12 times normal mass were the lymph nodes of the homolateral flank (forming a group of 2–4 nodes along a vein which descended from the side of the abdomen to the inguinal region). Sections showed large islands of granulomatous tissue made up mainly of masses of macrophages, with strands of lymphoid tissue between these and at the periphery. These strands contained many plasma cells. Appropriately treated sections showed the presence of many antibody-containing cells (identifiable as of the plasma cell series) within such lymphoid strands (Plate II).

The lumbar nodes were also many times enlarged and largely occupied by macrophage granulomata. Some plasma cells were present.

Other lymph nodes which were more remote from the injected foot-pad had much less macrophage tissue. The nodes of the contralateral flank region were only slightly enlarged and macrophage masses were absent. The main feature here was an intense plasma cell infiltration in the medullary strands, and tissue sections treated to demonstrate antibody showed large numbers of antibody-containing cells in this situation (Plate III).

Other lymph nodes remote from the site of injection, such as the thoracic and the cervical, also had many antibody-containing cells in colonies around the medullary vessels.

Liver.—The only histological change was the presence of small collections of lymphocytes, with scanty plasma cells, in the portal spaces. In appropriately stained frozen sections an occasional antibody-containing cell could be found in such areas.

Lungs.—Numerous sub-miliary granulomata were present, mainly subpleurally, and composed principally of macrophages with epithelioid characteristics and giant cells. Eosinophiles were also present; there were few lymphocytes and plasma cells. Appropriately treated sections revealed a few antibody-containing plasma cells.

Spleen.—Groups of macrophages were present in the medulla and within the sinuses. Colonies of antibody-containing cells identifiable as plasma cells were found grouped around medullary vessels.

Table I gives in simplified form the approximate relative densities of macrophages and antibody-containing plasma cells at various sites of the injected guinea-pig.

TABLE I.—*Approximate Density of Macrophages and Antibody-containing Plasma Cells in Different Tissues of the Immunised Guinea-pig*

	Macrophages.	Anti-ovalbumin-containing plasma cells.
Local granuloma of injected foot	+++	±
Popliteal lymph node	+++	±
Homolateral flank lymph node	++	++
Lumbar lymph node	++	±
Contralateral flank lymph node	±	+++
Cervical lymph node	±	++
Lung granulomata	+++	±
Liver	±	±
Spleen	+	+

Incorporation of Radioactive Amino-acids into Antibody and γ-Globulin Fraction 'in Vitro'

Fig. 2 records the incorporation of ^{14}C-glycine into the anti-ovalbumin and γ-globulin fractions at suitable time intervals by lymph nodes from the flank of immunised guinea-pigs. It can be seen that after an initial lag period the rates of

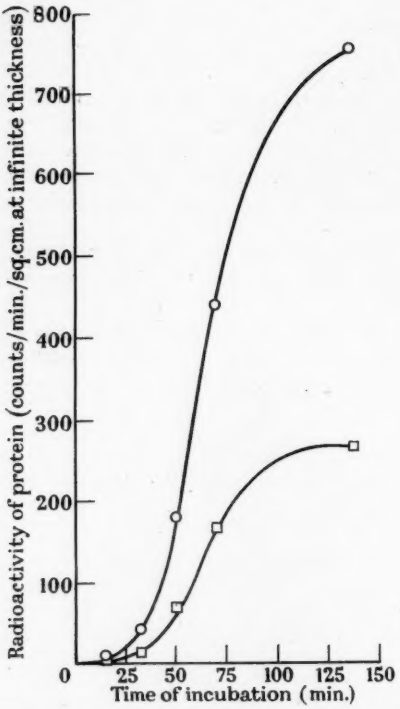


FIG. 2.—Time curve showing the incorporation of ^{14}C-glycine into anti-ovalbumin and γ-globulin fractions. ⊙ anti-ovalbumin; □ γ-globulin fraction. Sliced lymph nodes from the homolateral and contralateral flank of immunised guinea-pigs were incubated at 37° in an isotonic salt medium containing 0·3 per cent glucose, 2·5 μc ^{14}C-glycine and 1·7 mg. of an amino acid mixture; the gas phase was 5 per cent CO_2/95 per cent O_2.

incorporation of both anti-ovalbumin and γ-globulin fractions remain steady over a 2-hr. period, and then rapidly fall off. The term "γ-globulin fraction" refers to the salt-precipitated protein (*vide* Materials and Methods) which remains after separation of all precipitable anti-ovalbumin. This was shown by electrophoresis not to be homogeneous and to contain the slowest moving components of guinea-pig serum.

Antibody Production in Vitro *by the Local Granuloma, the Homolateral and the Contralateral Flank Lymph Nodes*

As seen from Table II the lymph nodes of the flank on both sides actively incorporated ^{14}C-glycine into anti-ovalbumin during the 4-hr. period of incubation *in vitro*. On the other hand, the granuloma of the injected foot had only 3–10 per cent of the activity shown by the homolateral flank node. Also, it is apparent that the radioactivity of the anti-ovalbumin from the contralateral was 2–3·5 times that of the homolateral flank lymph nodes.

Reference to the histological data (see also Table I) shows that whereas the granuloma of the foot consisted mostly of a proliferation of macrophage elements with only scanty plasma cells, the nodes of both flank regions included large numbers of antibody-containing plasma cells (Plates II and III). Moreover the density of such plasma cells was greater in the contralateral than in the homolateral flank nodes. These facts are compatible with the conclusions that the macrophages do not produce antibody, and that the demonstrated *in vitro* antibody production is in direct relation to the density of antibody-containing plasma cells in these tissues.

Incorporation of ^{14}C-glycine into the γ-globulin fraction followed a similar pattern (Table II). Thus the γ-globulin fraction isolated after 4-hr. incubation of the contralateral gland was 2–3 times more radioactive than the γ-globulin of the homolateral flank glands. However, the local granuloma would appear to form the γ-globulin fraction more actively than the anti-ovalbumin, since it was found to have up to 30 per cent of the activity of the same mass of homolateral flank gland.

TABLE II.—*Relative Ability of Homolateral and Contralateral Flank Lymph Nodes and Local Granuloma to form Anti-ovalbumin and γ-Globulin* 'in Vitro'

Radioactivity: (counts/min./sq. cm.)/0·1 g. tissue incubated

Expt.	Homolateral lymph node in flank.		Contralateral lymph node in flank.		Foot-pad		Contralateral node / Homolateral node	
	Anti-ovalbumin.	γ-globulin.	Anti-ovalbumin.	γ-globulin.	Anti-ovalbumin.	γ-globulin.	Anti-ovalbumin.	γ-globulin.
1*	384	157	907	450	7·8	33	2·36	2·87
2	170	81	590	230	7·9	31	3·45	2·85
3*	125	90	265	185	—	—	2·12	2·05
4	165	110	350	350	17	33	2·12	3·18

* 2 animals pooled.

Sliced tissues from immunised guinea-pigs were incubated for 4 hr. at 37° in the isotonic salt medium containing 2·8 μc ^{14}C-glycine, glucose and a complete amino acid mixture in a total volume of 4 ml. 5 per cent CO_2/95 per cent O_2 was circulated through the system.

Antibody and γ-Globulin Production by a Variety of Tissues of Immunised Guinea-pigs during a Period of Incubation ' in Vitro '

Table III gives data concerning the relative capacity of unit mass of a variety of guinea-pig tissues to incorporate ^{14}C-glycine into anti-ovalbumin and γ-globulin during 3 hours' incubation *in vitro*.

TABLE III.—*Radioactivity of Antibody and γ-Globulin Fractions after Incubation of Tissue Slices with ^{14}C-Glycine*

Tissue.	Weight of organ (g.).	Weight of tissue incubated (g.).	Radioactivity: (counts/min./sq. cm.) /0·1 g. tissue incubated		Density of antibody-containing cells*
			Anti-ovalbumin.	γ Globulin fraction.	
Homolateral flank lymph node	0·6	0·38	967	203	45·1
Contralateral flank lymph node	0·05	0·03	815	300	31·7
Cervical lymph node	0·1	0·08	880	632	15·2
Lumbar lymph node	0·3	0·2	39	23	6·4
Spleen	0·7	0·6	53	133	2·4
Lung	2·6	0·99	2·3	22	0·8
Red bone-marrow	3[a]	0·17	370	425	1·8
Kidney	3·8	1·0	2·5	26	0·1
Liver	16·4	1·0	2	<2	0·1
Thymus	0·25	0·21	—	9	0·1
Placenta	2·0	0·99	—	<2	0
Local granuloma in foot pad	>0·5	0·45	55	73	1·4

* Expressed as numbers per unit microscope field (0·0324 sq. mm.) of 4 μ frozen section.
[a] Approximate figure—taken as about 1 per cent of body weight.

These results are the mean of two experiments with guinea-pigs 79 and 80, immunised in foot pad. The minced organs were incubated *in vitro* for 3 hr. at 37° in an isotonic salt medium containing 0·3 per cent glucose, 1·7 mg. complete amino acid mixture and 2·5 μc ^{14}C-glycine.

Different weights of tissue were incubated, depending on their availability and activity. Thus more of the inactive tissues was incubated in order to increase radioactivity to more easily recordable levels. The results of the radioactivity determinations using different tissues are made comparable by expressing radioactivity of the protein in counts/minute/sq. cm. for 0·1 g. of tissue incubated. It had previously been established that the radioactivity of the protein formed was proportional to the weight of tissue incubated. Activity is also recorded as a percentage of that shown by the homolateral flank lymph nodes (Fig. 3).

The different tissues showed a wide range of activities. The highest radioactivity measurements per unit weight of tissue were attained by a range of lymph nodes, all of which are remote from the site of injection. The low activity of the lumbar nodes correlated with their relatively low content of antibody-containing plasma cells. These nodes are enlarged, but this is due to proliferation of macrophage elements. As described in the histological section, the other remote lymph nodes all contain dense proliferations of antibody-containing plasma cells. The bone marrow incorporated ^{14}C-glycine into anti-ovalbumin at one-third of the rate of the same mass of homolateral flank lymph node. Some activity was manifested by the spleen, but all other organs tested (lungs, liver, kidney, placenta and thymus) showed a negligible amount of incorporation of ^{14}C-glycine into the anti-ovalbumin fraction.

The results of counting antibody cells within appropriately stained sections of samples of the above tissues are shown in the last column of Table III. A fairly good correlation was shown in most instances between the *in vitro* activity of the tissue in the incorporation of radioactive amino acids into the anti-ovalbumin and the density of antibody-containing cells.

The general pattern of relative activities of the various tissues in incorporating ^{14}C-glycine into the γ-globulin fraction is similar to the above (Fig. 3). On the whole tissues which can form specific antibody also appear to form γ-globulin. The interpretation is somewhat limited by the lack of homogeneity in this fraction, and only broad conclusions are justified. It would appear that certain tissues form γ-globulin more actively than anti-ovalbumin (Fig. 3). This is strikingly apparent in the cervical lymph nodes, the local granuloma, the spleen and bone marrow.

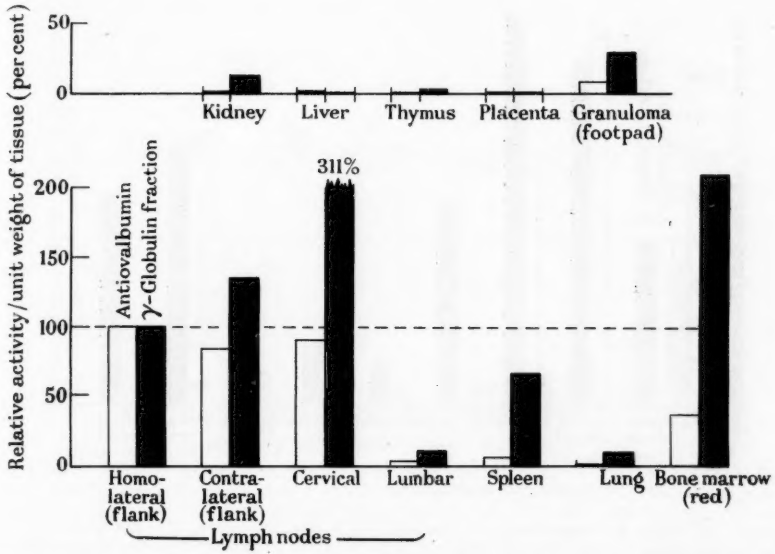


FIG. 3.—Data from guinea-pigs No. 79 and 80 (Table III) showing the relative ability of various tissues to incorporate ^{14}C-glycine into anti-ovalbumin and γ-globulin fractions *in vitro*. White columns: anti-ovalbumin; black columns: γ-globulin fraction. Tissues incubated for 3 hours at 37° in isotonic medium containing glucose, amino acid mixture and 2·5 μc of ^{14}C-glycine. Activity is represented by radioactivity of the protein fractions (counts/min./sq. cm. at infinite thickness) per 0·1 g. of tissue incubated. The activity of the homolateral flank lymph node was taken arbitrarily to represent 100 per cent activity.

Fig. 4 gives the result of another experiment with a greater number of lymph nodes. Some variation occurs from animal to animal in the relative activities of certain nodes. Thus if the various activity figures are referred to the arbitrarily chosen standard of the homolateral flank nodes some variation is apparent in the relative activities of the contralateral flank and cervical lymph nodes. The activity per 0·1 g. incubated contralateral flank node was strikingly high in the experiment shown in Fig. 4: the activity of the homolateral popliteal node was very low. The latter is the regional lymph node which immediately drains the injected foot with its depot of antigen. The appearance of tissue sections was one of an intense proliferation of macrophages which almost completely excluded the normal

lymphoid elements, and antibody-containing plasma cells were extremely few.

The results of counting antibody-containing cells within appropriately treated sections of samples of the above tissues are shown in Table IV. Having regard to the inaccuracies of the method, which were particularly increased in the very small lymph nodes weighing less than 0·2 g., and of which only a small part could be spared for a histological investigation, the figures for the counts of antibody-containing cells reproduce broadly the pattern of the *in vitro* activities. However,

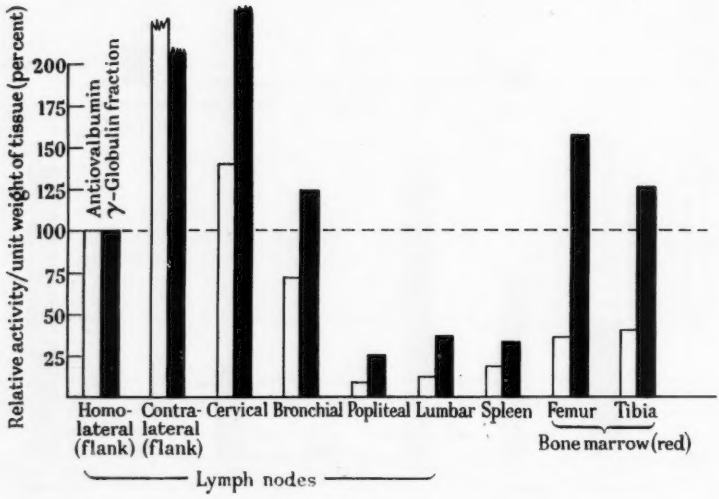


FIG. 4.—Data similar to those in Fig. 3 from guinea-pig No. 144.

the bone marrow appears more active *in vitro* than would be expected from the density of antibody-containing plasma cells. Thus in the experiments of Fig. 3 and 4 the bone marrow had more than one-third the activity of the homolateral flank lymph node and only 5–20 per cent of the number of antibody-containing plasma cells. Compared with the spleen the bone marrow contained fewer plasma cells and yet was 7 times as active *in vitro*.

TABLE IV.—*Density of Antibody-containing Plasma Cells in Various Tissues*

Number of cells per microscope field (area 0·0324 sq. mm.) Guinea-pig No. 144.

Homolateral flank lymph node	28·0
Contralateral flank lymph node (2)	42·3, 59·2
Cervical lymph node	29·2
Popliteal lymph node	0·9
Lumbar lymph node	1·4
Spleen	10·8
Bone-marrow (femur)	5·4

Estimation of the Relative Contributions made to the Production of Anti-ovalbumin and γ-Globulin by a Variety of Guinea-pig Tissues

Fig. 5 is an attempt to express the total activity of different tissues within an immunised guinea-pig, by taking into consideration the *in vitro* activity per unit weight of tissue as given in Table III and the total weight of tissue in the animal. The accuracy of this is limited by the fact that not all organs were studied. Also

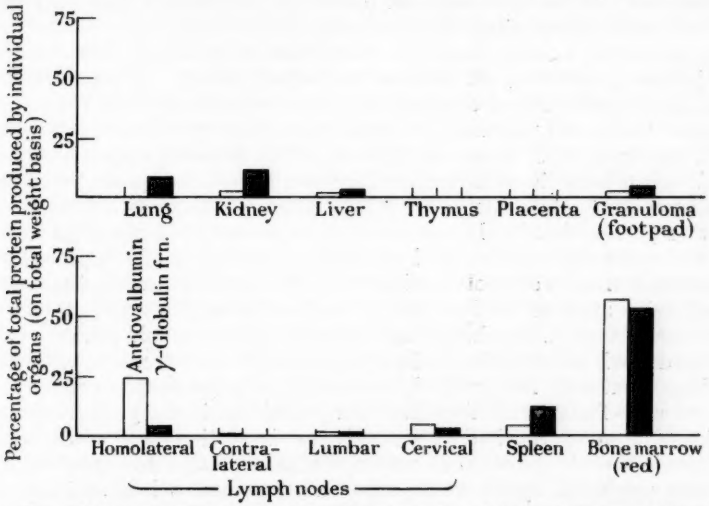


FIG. 5.—The relative proportion of anti-ovalbumin and γ-globulin fraction produced by individual organs. Black columns: γ-globulin fraction; white columns: anti-ovalbumin. Calculations are based on experimental findings described in Table III. Results are expressed as percentage of total anti-ovalbumin and γ-globulin produced by each of all the organs tested, on basis of its total weight and relative *in vitro* activity per 0·1 g. of tissue incubated.

bone marrow contains differing proportions of red and yellow marrow in various physiological conditions. The figure of 1 per cent of body weight taken here is based on the work of Nye (1931) who estimated that the red bone marrow formed 1 and 1·7 per cent of the total body weight of human beings and rabbits respectively.

As shown by Fig. 5, the main contribution to the total anti-ovalbumin production of the guinea-pig is made by the lymph nodes of the flank and by the bone marrow. The cervical lymph nodes and the spleen add a small amount (less than 10 per cent). The remaining tissues studied make negligible contributions or none at all.

DISCUSSION

The capacity of a variety of different lymph nodes, on a unit weight basis, to synthesise anti-ovalbumin *in vitro*, varied over a wide range of values and this could be correlated with the numbers of antibody-containing cells within these. Since such cells are identifiable as members of the plasma cell family (White *et al.*, 1955) further evidence is provided for the rôle of these cells as producers of specific antibody globulin.

There has been much discussion whether incorporation of a ^{14}C-amino-acid into a protein *in vitro* represents protein synthesis or whether the protein becomes radioactive by adsorption on existing protein or by a non-specific exchange reaction. Granuloma tissue from rabbits immunised with ovalbumin was found (Askonas and Humphrey, 1955) to incorporate ^{14}C-glycine into the anti-ovalbumin during incubation *in vitro*, but not into rabbit pneumococcal antibody added at the start of incubation. Thus it may be taken that incorporation of ^{14}C-amino-acids into the soluble antibody and γ-globulin fractions occurs only when the incubated tissue is producing these proteins.

The numerous studies based on extraction of antibody from tissues after immunisation procedures all possess the disadvantage of possible confusion between local production of antibody and local concentration of antibody formed elsewhere. One is left wondering whether the studies of Westwater (1940*a*,*b*) and Freund, Schryver, McGuiness, and Geitner (1952), showing high concentrations of antibody at the local site of injection of antigen in Freund-type adjuvant mixtures, really denote local production of antibody. There is a suggestion from the work of Westwater (1940*a*) that local concentration was actually occurring, for in some animals from which the local lesions had been removed antibody appeared sooner in the circulation than in controls in which the lesion was left intact, *i.e.*, in the latter the local lesion appeared to function by extracting antibody from the circulation until it was saturated. In the present animals the 3-week-old lesions of the footpad showed much extracellular antibody associated with adventitia of blood vessels and collagen but very few antibody-containing cells (or plasma cells).

The present findings are that the local granuloma in the guinea-pig under the condition of immunisation had a low relative *in vitro* activity and would be expected to contribute little to the animal's production of antibody. In the rabbit, the local granuloma produced by Freund's adjuvant possesses a low activity per unit weight of tissue when compared with the antibody-synthesising activity of lymph nodes ; nevertheless, on the basis of its total weight the granuloma appears to contribute an appreciable proportion of the antibody produced by the whole animal (Askonas and Humphrey, 1955). It is possible that conditions such as dosage of antigen, species of experimental animal and site of injection may greatly influence the rate of production of antibody at the local site of injection of antigen, and the proportion which this contributes to the antibody produced in the whole animal.

The low numbers of antibody-containing cells found in the other tissues of the body (liver, lungs, kidney, spleen, thymus) correlated well with the low or negligible *in vitro* activity for anti-ovalbumin synthesis, with the exception of the bone-marrow. The few experiments which have included the study of this tissue have failed to show as many plasma cells or antibody-containing cells as would be expected from the relatively high *in vitro* activity. This discrepancy is being investigated. The technical difficulties of the morphological studies are greater with the bone marrow than with other tissues. The matter is of some importance, since by assuming the weight of bone marrow to equal about 1 per cent of the total body weight, this tissue could be regarded as an important site of the antibody formation of the whole animal.

While the present investigation provides evidence that specific antibody is formed by members of the family of plasma cells, the cellular site of production of γ-globulin is still far from clear. According to the Mudd-Haurowitz hypothesis the

presence of antigen diverts the activities of the cell normally producing γ-globulin to form specific antibody. Since the numerous plasma cells found in immunised animals make their appearance in response to the specific stimulus of antigen the production of " normal " γ-globulin as envisaged by the Mudd-Haurowitz theory cannot apply to these newly differentiated cells. The reconciliation of this theory with morphological facts is only possible if a precursor of plasma cells is responsible for synthesis of " normal " γ-globulin and if the morphological change implicit in the development of the family of plasma cells is a manifestation of increased rate of protein synthesis. Unfortunately the nature of the precursor of the plasma cell family is obscure.

Alternatively, the plasma components classed as " normal " γ-globulin may consist of a collection of small amounts of specific antibody (Hughes, 1954). The γ-globulin fraction is known to be heterogeneous (Porter, 1955). The morphological implication of this hypothesis would be that all γ-globulin production would be associated with plasma cells. This view receives support from the findings that patients suffering from agammaglobulinaemia do not form plasma cells when stimulated with antigens (Good, 1955 ; Gitlin, 1955) which suggests that the essential defect of agammaglobulinaemia is located in the same system of cells.

Conclusions about γ-globulin formation based on the present results are limited by the nature of antigen administration and by the impurity of the γ-globulin fraction. They do show, however, that no γ-globulin is synthesised by the liver. This supports the evidence of Miller and Bale (1954) obtained by the use of perfused liver preparations. Similarly, thymus and placenta show no activity. However, many other tissues appear to contribute to the production of γ-globulin to varying extents. On the whole, formation of the γ-globulin fraction in various tissues follows a pattern similar to that found for the synthesis of anti-ovalbumin. Tissues such as the homolateral and contralateral lymph nodes of the flank, the cervical lymph nodes and the bone marow, which actively incorporate ^{14}C-glycine into anti-ovalbumin, are also active in forming the γ-globulin fraction (which contains the γ-globulin components not precipitable with ovalbumin.)

The low *in vitro* activity of the local granuloma, and of the homolateral popliteal and lumbar lymph nodes, which in these animals are largely composed of macrophages, would certainly indicate that the latter are not responsible for production of γ-globulin. Furthermore it is striking that certain organs can incorporate the radioactive amino acid more rapidly into the γ-globulin fraction than into the anti-ovalbumin (*i.e.*, cervical lymph nodes, bone marrow and spleen). These tissues must be important contributors to the production of γ-globulin found normally in the plasma.

SUMMARY.

Guinea-pigs were injected in the foot-pad with a single dose of ovalbumin in a Freund-type adjuvant mixture and three weeks afterwards various tissues were examined for their content of antibody-containing plasma cells and their ability to incorporate ^{14}C-glycine into the anti-ovalbumin and the remainder of the γ-globulin during a period of incubation *in vitro*.

The granuloma at the site of injection of the antigen mixture, and the immediate draining lymph node (popliteal) which were composed mostly of macrophages and had few antibody-containing plasma cells were relatively inactive in the synthesis of anti-ovalbumin *in vitro*.

The lymph nodes more remote from the site of injection were extremely active in incorporating ^{14}C-glycine into the anti-ovalbumin. In most cases, the highest activity was found in the lymph nodes of the contralateral flank.

Organs other than lymph nodes had low *in vitro* activity with the exception of the spleen and bone marrow. If this activity of the bone marrow were uniform throughout the red marrow of the whole animal this would be a major contributor to the total antibody.

A good correlation existed throughout the lymph nodes and the other organs tested (with the exception of the bone marrow) between the number of antibody-containing plasma cells seen in tissue sections and the ability of unit weight of the different tissues to form anti-ovalbumin *in vitro*.

The authors are indebted to Dr. J. H. Humphrey for suggesting this investigation. This work owes much to the excellent technical assistance of Miss M. T. Smith, Miss P. Thorne and the late Mr. F. V. Welch.

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NORMAL SERUM INHIBITORS OF INFLUENZA VIRUS FLOCCULATION.

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In a previous paper (Belyavin, 1955) it was reported that influenza virus elementary bodies can be specifically agglutinated by immune rabbit sera and that this reaction may be blocked by inhibitory substances present in virus-infected allantoic fluids. Apart from the intrinsic interest of this inhibitory effect, hitherto unobserved in other specific flocculations, the possibility that such blocking agents may be present in the sera of convalescent humans and experimentally immunised animals is clearly of practical importance. The occurrence of such flocculation inhibitors in immune sera was considered probable for two reasons: first, that the sera of many animal species contain substances which inhibit the haemagglutinating activity of influenza viruses and, second, because it had been noticed that certain sera which, on the basis of the immune status of the donor animal should have possessed strong flocculating activity, were in fact remarkably poor in this respect.

This paper described the results of experiments designed to ascertain the presence and species distribution of serum flocculation inhibitors and their relationship to the normal haemagglutination inhibitors of the influenza virus.

MATERIALS AND METHODS.

Influenza virus strains.

Type A strain PR8, Type A prime strains FMI, MW, ASH and Type B strain LEE were used for all the experiments. The A-prime strains MW and ASH were isolated in this laboratory in 1951 and 1953 respectively. The strains were propagated by the allantoic inoculation of 11-day old embryonated hens' eggs with 0·2 ml. of suitably diluted seed virus, the infected allantoic fluids being harvested after 40 hours' incubation at 35°. All test antigens were purified by two cycles of absorption and elution on human group O cells by a standard technique (Sheffield, Smith and Belyavin, 1954).

Haemagglutination titrations.

Serum haemagglutination inhibitor and virus titres were measured by means of the haemagglutination test using a photo-electric densitometer (Belyavin, Westwood, Smith and Please, 1951).

Sera.

Flocculating antisera were prepared by the intravenous immunisation of rabbits with two doses of virus given at an interval of twenty days, the second dose being about ten times larger than the first in terms of haemagglutinin content. These sera were tested for flocculating anti-bodies by the technique previously described (Belyavin, 1955).

Titration of flocculation inhibitor in serum.

Non-immune serum was diluted serially in twofold steps with normal saline, and 0·15 ml. aliquots of each dilution pipetted into Dreyer's tubes: 0·15 ml. of specific flocculating antiserum, homologous for the virus test strain, was then added, diluted so as to give about s + agglutination in 4–5 hr. in the absence of inhibitor. In addition, a control tube was included containing 0·15 ml. saline and 0·15 ml. flocculating serum. Each tube was then flicked lightly with the finger to mix the contents, and 0·3 ml. of suitably diluted virus antigen (containing approximately 50 agglutinating doses) was added. The tubes were then placed in a water bath at 37° and read in a viewing box after 4½–5 hr. incubation, by which time the control tube showed well marked flocculation. The inhibitor titre of the serum was taken as the highest final dilution after mixing with flocculating serum, giving tr + flocculation.

EXPERIMENTAL.

The Heat Sensitivity of Flocculation Inhibitors and their Species Distribution.

Samples of blood were collected from laboratory animals and human volunteers, allowed to clot, and the serum separated and frozen immediately. The sera obtained from rabbits, mice and guinea-pigs were pooled in lots involving up to a dozen animals. The samples obtained from humans, fowls and horses were retained as individual sera. None of the laboratory animals had been previously immunised or infected with influenza viruses.

The sera, both freshly thawed and after heating at 56°/1 hr., were tested for flocculation inhibitor against different strains of influenza virus by the technique described above. The results of three experiments are summarised in Table I. It is clear that the inhibitor effect of the sera is directed impartially, though to a varying degree, against virus strains of different antigenic constitution, and is readily abolished by heating. These facts excluded the possibility that the inhibition may be ascribed to the action of a " blocking " antibody, itself unable

TABLE I.—*The Flocculation Inhibitor Titres of Different Animal Sera before and after Heating at 56° for 60 minutes.*

		Sera.											
		Pooled rabbit.		Pooled g-pig.		Human.		Pooled mouse.		Fowl.		Horse.	
Expt. number.	Test strain.	unh.	heat.	unh.	heat.	unh.	heat.	unh.	heat.	unh.	heat.	unh.	heat.
1	PR8	5	0	0	0	0	0	—	—	—	—	—	—
	FMI	0	0	0	0	7	0	—	—	—	—	—	—
	MW	40	0	10	0	>160	0	—	—	—	—	—	—
	ASH	40	0	10	0	160	0	—	—	—	—	—	—
	LEE	15	0	5	0	80	0	—	—	—	—	—	—
2	PR8	0	0	15	0	7	0	15	0*	0	0	—	—
	ASH	15	0	30	0	80	5	60	0*	5	0	—	—
	LEE	5	0	15	0	60	0	30	0*	0	0	—	—
3	PR8	7	0	40	0	160	0	160	0	15	0	10	0
	ASH	160	0	120	0	>320	0	160	0	30	0	0	0
	LEE	5	0	15	0	10	0	15	0	0	0	0	0

0 = No sign of flocculation at the lowest dilution used — 1/5.
* Lowest dilution 1/10.

Figures in all Tables give flocculation inhibitor titres.

to flocculate virus. It is also evident that the inhibiting material is widely distributed amongst animal sera, though the average content may vary characteristically from species to species.

Effect of Receptor-destroying Enzyme and Trypsin on Flocculation Inhibitor.

It was of interest to examine the susceptibility of the flocculation inhibitors to certain enzymes, such as the receptor-destroying enzyme (RDE) of *Vibrio cholerae* and trypsin, in order to compare them with the known enzyme sensitivities of the serum haemagglutination inhibitors. To this end normal human and normal rabbit sera, suitably diluted with physiological saline, were treated for 1 hr. with *Vibrio cholerae* filtrate at 37°. As the activity of the RDE was to be controlled by titrating untreated and treated sera in parallel for haemagglutination inhibitor against heated LEE virus, and furthermore, as the presence of active RDE in the flocculation test materials might be deleterious, it was decided to inactivate the enzyme by heating at 56° for 20 min. in the presence of 2 per cent sodium citrate. In view of the known thermolability of the flocculation inhibitors this heat inactivation process necessitated the introduction of an additional control. In the final experimental design, therefore, 3·5 ml. of diluted human or rabbit serum were pipetted into each of three tubes. To one tube was added 1 ml. cholera filtrate and to the other two, 1 ml. saline. All the tubes were maintained at 37° for 1 hr., at the end of which time 0·5 ml. 20 per cent sodium citrate was added to each and the tube containing filtrate and one other, designated the " heated control ", were heated at 56°/10 min. The final dilution of serum was 1/2·5. All the mixtures were then titrated for flocculation inhibitor content. The results of such experiments are summarised in Table II.

TABLE II.—*Summary of Experiments showing Action of RDE on Serum Flocculation Inhibitor.*

Test strain.	Human serum. With Untr.	Human serum. With RDE.	Human serum. Heated control.	Rabbit serum. With Untr.	Rabbit serum. With RDE.	Rabbit serum. Heated control.
ASH	>160	30	30	—	—	—
LEE	120	30	20	—	—	—
ASH	80	8	5	30	10	8
LEE	60	8	5	30	5	5

The sera treated with RDE and " heated control " sera were heated at 56°/20 min. in these experiments to inactivate the enzyme.

Little difference was found in the inhibitor content of the " active RDE " and " heated control " tubes, but both contained less flocculation inhibitor than the tube which had not been heated at 56°. It could be presumed therefore that RDE had little effect on the flocculation inhibitor, any change in concentration being due to the heat inactivation treatment. In order to confirm this point the method was modified by applying heat inactivation only to a small aliquot of treated serum to be used for the haemagglutination inhibition tests, therefore doing away with the " heated control "' mixtures.

The results so obtained still showed some inactivation of the flocculation inhibitor by the RDE and to exclude the action of some other (possibly proteolytic) enzyme present in the crude cholera filtrates, further experiments were performed in parallel using RDE purified by adsorption and elution from human group O cells (Burnet and Stone, 1947). Treatment with crystalline trypsin at a concentration of 20 mg./ml. undiluted serum was also carried out under the same conditions and by the same technique. The results are summarised in Table III.

TABLE III.—*The Effects of Purified and Unpurified RDE and Crystalline Trypsin on Serum Flocculation Inhibitor.*

Expt. number.	Test strain.	Human serum treated with:				Rabbit serum treated with:	
		Nil.	Crude RDE.	Purif. RDE.	Cryst. trypsin.	*Nil.*	Crude RDE.
1	ASH	160	80	—	—	60	5
	LEE	120	40	—	—	40	30
2	LEE	80	40	80	5	—	—
3	LEE	120	80	120	—	—	—
4	LEE	30	—	—	<5	—	—

Citrate Effect.

In the experiments with RDE it was noted that the tubes containing the lowest dilutions of serum would frequently show slight partial flocculation at 1½–2 hr. and, with the further lapse of time this flocculation increased and tended to spread towards the higher dilutions, leaving only a narrow range of inhibition. As this was only observed in the lower dilutions of those serum mixtures which contained sodium citrate, it was suspected that this salt was responsible for the effect. To test this normal human serum was diluted 1/2·5 with physiological saline, divided into two equal volumes, and enough 20 per cent sodium citrate was added to one half to give a final concentration of 2 per cent. An equivalent volume of saline was added to the other half. The sera were then titrated for flocculation inhibitor against LEE virus unheated and heated at 56°/30 min. The heated virus was used to exclude possible sensitisation of the inhibitor to the virus enzyme by the citrate. The results of such an experiment, showing the abolition of the flocculation inhibition by sodium citrate, are shown in Table IV.

TABLE IV.—*The Effect of Sodium Citrate on the Inhibition of Direct Virus Flocculation by Normal Serum.*

Time of reading test.	Test strain.	Flocculation inhibitor titre of normal human serum:	
		+ saline.	+ sodium citrate (2%).
1½ hr.	LEE unheated	32	32
	LEE heated	64	48
4½ hr.	LEE unheated	16	<4
	LEE heated	24	<4

DISCUSSION.

The presence in rabbit or human sera of specific " blocking " antibody was a theoretically possible explanation of the inhibition of influenza virus flocculation. This is readily excluded by the results presented here, for the apparent thermolability of the inhibitory substance, its marked susceptibility to trypsin and lack of serological specificity show it to be quite unlike specific antibody. Furthermore if it be granted, as these experiments seem to show, that the inhibitor found in the sera of all the animal species tested is essentially the same substance, this is further evidence against the antibody hypothesis, for none of these species except man is normally susceptible to influenza in nature. It is not possible, however, to exclude entirely a relationship of this inhibitor to so-called " natural " antibody.

The lack of susceptibility of the flocculation inhibitor to RDE, its apparent thermolability and its greater activity against recently isolated A-prime strains, such as ASH, suggest identity with the β haemagglutination inhibitor of influenza virus first observed in mouse and rabbit sera (Chu, 1951: Smith, Westwood and Belyavin, 1951). It has been found, however (Belyavin, unpublished) that human serum contains very little, if any, β inhibitor, although containing high titres of flocculation inhibitor, so that it seems unlikely that these two inhibitors are identical.

Although the data presented here throw no light on how flocculation inhibition is effected, it is useful to make some tentative formulation of possible mechanisms. Results derived from absorptions of immune sera strongly suggest that the flocculation inhibitor combines primarily with the virus and not with the flocculating antibody. It has also been shown that flocculation of the virus is brought about by union of specific antibodies with the virus particles (Belyavin, 1955). The simplest explanation is that inhibition is brought about by prevention of the antibody-virus union, but it is equally possible that both specific antibody and inhibitor unite with the virus. In the latter case aggregation might be prevented by large molecules of inhibitor projecting from the virus surface and overcoming the effect of specific antibody by virtue of their large electrostatic charges. The implications of these alternative hypotheses extend beyond the immediate phenomenon of flocculation inhibition. It has been shown that a normal human serum treated with RDE preserves its flocculation inhibitor intact whilst losing its haemagglutinin inhibitor. This indicates that the flocculation inhibitor does not unite directly with the virus haemagglutinin receptors. The two hypotheses put forward above, therefore, imply two alternative modes of combination of flocculating antibody molecules with the virus surface, either direct union with haemagglutination receptors, or attachment to other sites not connected with haemagglutination phenomena.

SUMMARY.

The direct flocculation of influenza virus by immune rabbit sera can be inhibited by a substance present in the normal sera of several animal species.

The inhibitor is thermolabile and is destroyed by crystalline trypsin but not by RDE. Its effect is reversed by sodium citrate.

The flocculation inhibitor is distinct from the haemagglutination inhibitor of normal sera.

Alternative hypotheses of the mechanism of flocculation inhibition are discussed.

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THE METABOLISM OF FLUORESCEIN-LABELLED AND UNLABELLED EGG-WHITE IN THE RENAL TUBULES OF THE MOUSE

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THE parenteral administration of foreign proteins, of sufficiently low molecular weight to pass through the glomerular membrane, produces a variety of morphological changes in the renal parenchyma. Native egg-white protein is particularly active, as shown by Oliver (1948), and the changes occurring in rat kidney have been very thoroughly described by Oliver, Macdowell and Lee (1954*a*). Until recently there has been no general agreement on the mode of absorption of proteins which appear in the glomerular filtrate. In particular, the " hyaline " droplets which characteristically appear at some stages of resorption have, in the past, been variously interpreted as the original protein, as degenerative phenomena or as secretion droplets of new material. These interpretations are still held by some authorities.

More recently Zollinger (1950) and Rüttimann (1951), using phase contrast microscopy, have described the intracellular granules and droplets in the cells of the proximal convoluted tubules as altered mitochondria. The work of Oliver *et al.* (1954*a*) and Oliver, Moses, Macdowell and Lee (1954*b*), combining morphological studies and conventional histochemical methods, has produced strong support for this thesis, and Oliver, Straus, Kretchner, Lee, Dickerman and Cherot (1955) regard the droplets as a "combination of the excess protein and mitochondrial substances and their enzymes ".

The electron microscope studies of Rhodin (1954) have confirmed the views of Zollinger, Rüttimann and Oliver in showing that, in the mouse, the changes produced by intraperitoneal injection of egg-white are confined to the " mitochondria of the first two-fourths of the proximal convoluted tubules ", but they have not upheld the views of Zollinger on the participation of the microsomes in the formation of granules. According to Rhodin, 7 hours after the injection of egg-white the mitochondria and a finely granular substance coalesce to large granules, while from 18–30 hr. after injection the cell is filled with these granules. Later, restoration of mitochondria takes place, in part from the large granules themselves.

These investigations, even those employing histochemical methods for protein, afford only indirect proof of the incorporation of protein into the droplets. More direct proof is offered by Straus and Oliver (1955) who have found that in kidney homogenates, prepared 18 hr. after the injection of egg-white, the highest content of egg-white by immunological assay is present in the fraction containing the droplets.

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The use of labelled proteins, which might be expected to provide more direct evidence, is derived from the work of Smetana (1947), who first employed coloured azo proteins for this purpose, and from that of Kruse and McMaster (1949) who used γ-globulin azo-coupled to the dye Echt saüre blau. Provided that the protein can be shown to retain its label throughout its existence *in vivo*, methods such as these would seem to offer a solution to the problem. However, from the work of the above authors and that of Gitlin (1950), it is evident that only the broad disposition of the injected protein can be traced. Four further possibilities exist: (1) *Chromogenic labelling* (the attachment to the protein of a small colourless group which is subsequently converted into a highly coloured dye); (2) *Fluorescence labelling* (the attachment to the protein of groups conferring the property of fluorescence in ultra-violet light; and (3) *Fluorescent antibody methods* (the tracing of an antigenic protein by attaching to it *in vitro* its fluorescein-coupled antibody). (4) *Isotope labelling*, followed by auto-radiography.

We have used, unsuccessfully, a method employing the first principle (briefly described in the section on technique) with which interpretation was no easier

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EXPLANATION OF PLATES

All figures represent 5 μ paraffin sections of mouse kidney.
All fluorescence exposures 20 min. unless otherwise indicated.

FIG. 1.—(Freeze-dried) 2 hr. after injection of labelled egg-white (unpurified). Shows specific fluorescence of tubules and "unspecific" fluorescence (not autofluorescence) of elastica. × 800.

FIG. 2.—(Freeze-dried) 4 hr. after injection of purified labelled egg-white (FEF). General view. Shows strong fluorescence of some groups of proximal tubules, weak or absent in others. Exposure 5 min. × 300.

FIG. 3.—(Freeze-dried) 30 min. after FEF. Strong fluorescence in tubular lumen, brush borders and around the tubular nuclei. × 800.

FIG. 4.—(Freeze-dried) 30 min. after FEF. Strong fluorescence of the connective tissues of the renal capsule. × 800.

FIG. 5.—(Freeze-dried) 4 hr. after FEF. Shows strong fluorescence of the brush border and diffusely fluorescent cytoplasm. Tubular lumina and blood vessels are also strongly positive. × 600.

FIG. 6.—(Cold formalin-fixed). As Fig. 5. Localisation of specific fluorescence is identical. Autofluorescence greatly increased. × 600.

FIG. 7.—(Freeze-dried). Antigen-antibody reaction, 2 hr. after injection of F.E. Nuclei and perinuclear rings show strong fluorescence. Brush borders also positive. × 800.

FIG. 8.—(Freeze-dried) 8 hr. after FEF. Strong fluorescence of lumina, brush borders, and in cytoplasm of tubule cells. Exposure 7 min. to show strongest localisations. × 600.

FIG. 9.—(Helly-fixed). As Fig. 8 but exposure 30 min. Shows same localisation of fluorescence but indicates strong quenching effect of Helly fixation. × 600.

FIG. 10.—(Freeze-dried) 8 hr. after FEF. Collecting tubules strongly positive. × 600.

FIG. 11.—(Helly-fixed) 8 hr. after FEF. Perinuclear masses are numerous. Iron haematoxylin. × 800.

FIG. 12.—(Freeze-dried) 18 hr. after FEF. Moderate even fluorescence of cytoplasm and brush border. Nuclei now free. Exposure 15 min. × 800.

FIG. 13.—(Freeze-dried, post-fixed Helly) 18 hr. after FEF. Fine intracellular droplets are present and these are therefore unlikely to be a fixation artefact. Iron haematoxylin. × 800.

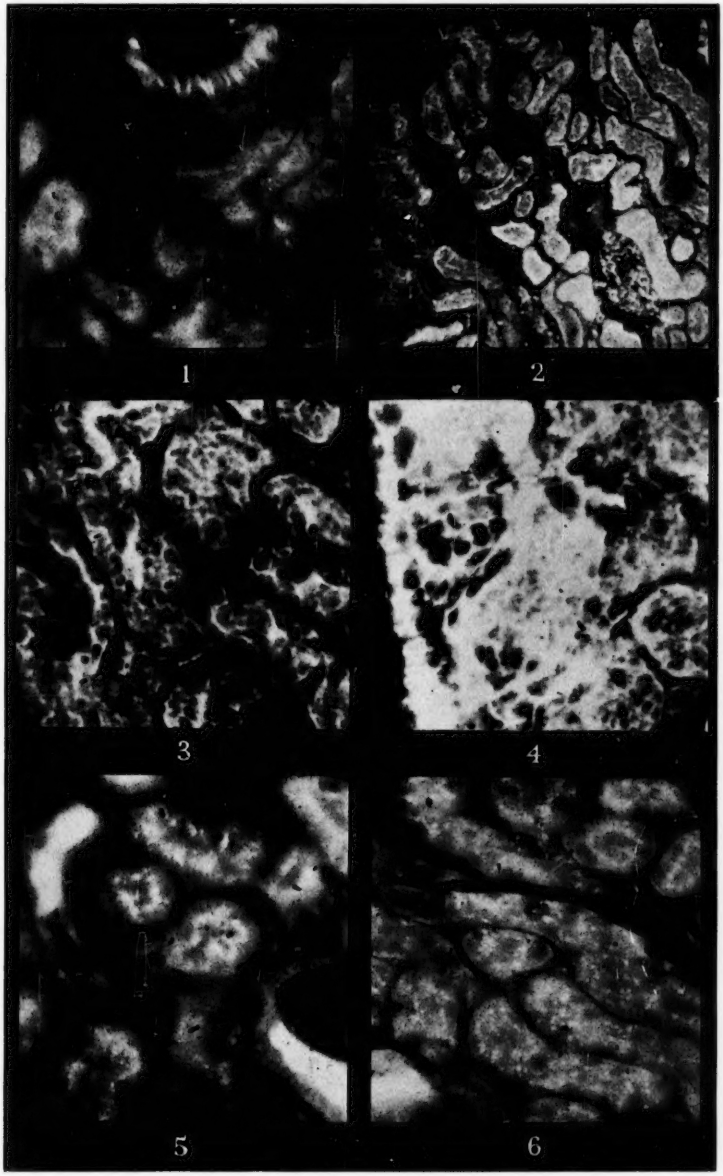
FIG. 14.—(Freeze-dried) 30 hr. after FEF. Fairly strong general fluorescence of the cytoplasm and still visible brush borders. Note strongest fluorescence outlining the basement membranes. × 800.

FIG. 15.—(Helly-fixed) 30 hr. after FEF. Rebuilding of mitochondria is taking place, but perinuclear and nuclear masses and droplets are still present. Iron haematoxylin. × 800.

FIG. 16.—(Freeze-dried) 8 hr. after FEF. Distal tubules free from specific (fluorescein) fluorescence. Blood vessels positive. × 600.

FIG. 17.—(Freeze-dried) 30 hr. after FEF. Distal tubules and Henle's loops show strong specific fluorescence; blood vessels negative. × 600.

FIG. 18.—(Cold formalin) 8 hr. after FEF. Shows strong "clotting" effect of this fixation. The masses are mainly confluent. Iron haematoxylin. × 800.



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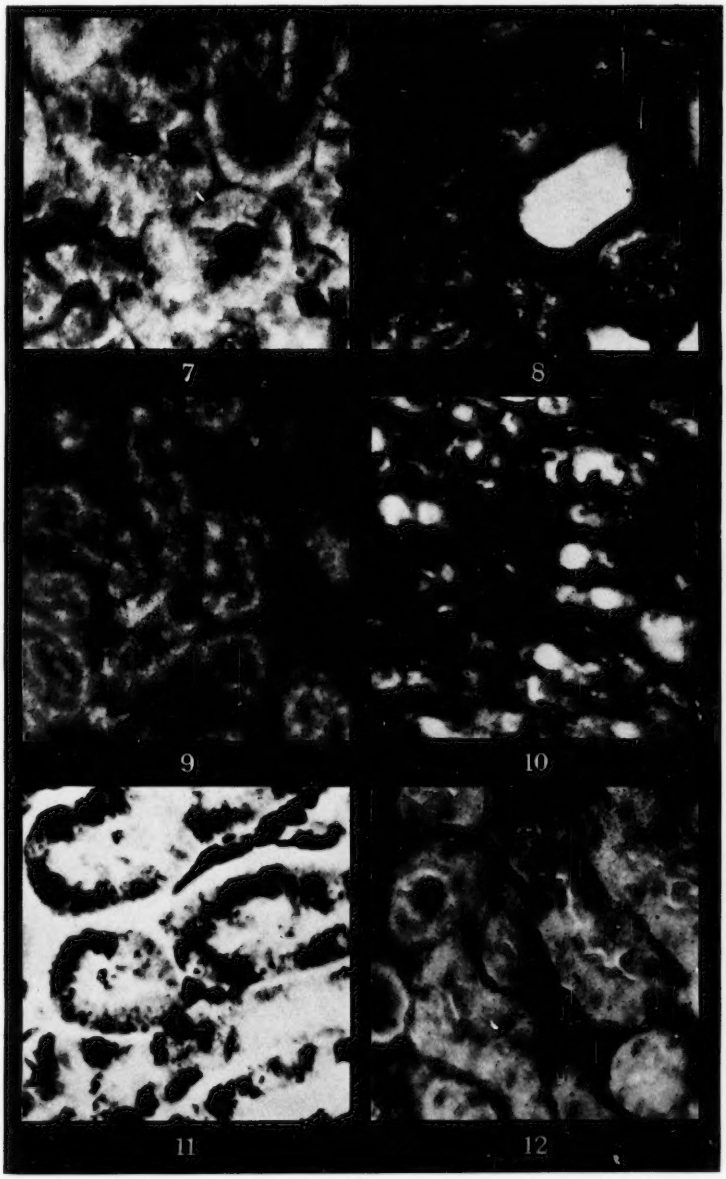

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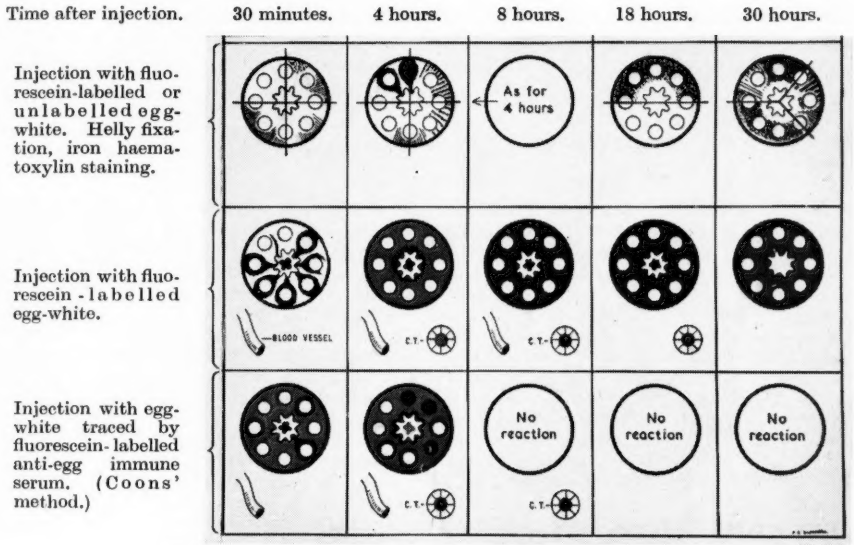

Diagram.—Egg-white-induced changes in the renal tubules.

than with the coloured proteins. The second principle has been employed by Schiller, Schayer and Hess (1952–53), whose critical work has established clearly the physical and immunological properties and the general usefulness of fluorescein-coupled protein markers, and the third by Coons and his associates (Coons, Creech, Jones and Berliner, 1942 ; Coons, Leduc and Kaplan, 1951), who have also shown conclusively that such labelling leaves proteins immunologically intact (Kaplan, Coons and Deane, 1950).

The results obtained by Coons appear to differ entirely from those of workers using non-fluorescence techniques and, to some extent from those of Schiller *et al.*, although the latter did not pursue the question very far. In order to throw some light on these differences we decided to apply the fluorescein marker and the fluorescent antibody techniques in parallel with conventional morphological and histochemical studies.

Studies by the fourth method, which we ourselves did not use, are considered in the discussion at the end of the paper.

MATERIAL AND METHODS

Injection Materials

(1) Fresh egg-white (FE) : Egg-white from fresh hen eggs was mixed with the same volume of physiological saline and filtered through gauze. Protein content 67 mg./ml. (2) Crystallised egg-white (CE) : Egg-white from fresh hen eggs, crystallised by the method of Kekwick and Cannan (1936) was dissolved in physiological saline. Protein content 60 mg./ml. (3) Fluorescein-conjugated egg-white : The stock solutions of FE and CE were coupled with fluorescein isocyanate by the method of Coons and Kaplan (1950) and dialysed at 4° against 0·5 M carbonate-bicarbonate buffered saline (pH 7·4) until the latter was free from fluorescence. Further purification of the fluorescein-conjugated fresh (FEF) and crystallised (CEF) egg-white solutions was carried out with acetone-dried mouse and pig liver powder, the undiluted dialysate being absorbed with liver powder without previous precipitation by ammonium sulphate or acetone. Egg-white solution (1 ml.) was mixed with 100 mg. liver powder, shaken gently at room temp. and centrifuged for 20 min. This procedure was repeated twice and the final supernatant was used for injection.

The injection of the unpurified dialysate (FEF or CEF) resulted in diffuse staining of all tissues with predilection for certain sites, such as the elastica of blood vessels (Fig. 1). No satisfactory explanation was forthcoming for this last result, which was quite distinct from the usual and well-known autofluorescence of elastic tissue. Previous blocking with phenol or naphthols of the specific groups in elastic tissue responsible for its affinity for various dyes (including fluorescein) failed to prevent uptake of the component of unpurified FEF or CEF which was absorbed by liver powder. This suggests at least that the fluorescein is not attached to the elastin in the way suggested by Weiss (1954), that is to say by hydrogen bonding through its phenolic groups.

(4) Naphthaldehyde-conjugated egg-white : This was prepared by coupling 3-hydroxy-2-naphthaldehyde (Weiss, Tsou and Seligman, 1954) with egg-white buffered to pH 10, followed by dialysis against carbonate bicarbonate buffer as above. Freeze-dried sections of kidney and liver containing the labelled egg-white were fixed in ethanol and treated with a diazonium salt at pH 7·4 (Fast Blue B). On chromatographic separation of similarly coupled whole serum the albumen fraction gave a strong black colour after treatment with the diazonium salt, but the maximum colour developed in the tissues was a very pale brownish-red.

Experimental Procedure

Groups of 5–6-week-old white female mice (20–22 g.) were used. These were chosen because recent work by Logothetopoulos and Weinbren (1955) has shown that naturally occurring protein-containing droplets are not found in the renal tubules of mice of this age and sex. The animals received "Research" mouse cubes and tap water *ad lib.* Each animal was given a single intraperitoneal injection of 2 ml. FE or CE or of the equivalent

amount of FEF or CEF (2·2–2·4 ml.). The animals were killed with coal gas 30 min., 2, 4, 8, 12, 18, 20, 24 and 48 hr. and 3, 5 and 10 days after injection. The organs were taken out as quickly as possible and cut into thin slices. Each slice was either immediately quenched in Arcton 6 (I.C.I. Ltd.) as recommended by Bell (1952), cooled to about − 150° with liquid nitrogen, and subsequently freeze-dried, or placed in cold 12 per cent buffered formalin or Helly's fixative. After paraffin embedding serial sections were cut at 5 μ, those from freeze-dried material being mounted on clean slides with finger pressure only. The freeze-dried sections were de-waxed in light petroleum and the tissues fixed by 30 min. immersion in absolute ethanol. In a few cases post-fixation of the freeze-dried sections was done with Helly.

Light microscopy.—For light microscopy sections prepared by each of the above methods were stained by Heidenhain's iron haematoxylin, Gram's method, methyl green/pyronin (Trevan and Sharrock, 1951), a trichrome periodic acid-Schiff method, and by Schiff's reagent after oxidation with aqueous periodic acid. Some sections were treated before staining with crystalline ribonuclease (0·5 mg./10 ml. 0·1 M veronal acetate buffer at pH 8·5). Buffer was used in order to obtain results comparable with those of Oliver *et al.* (1954*b*).

Coons' method.—For the tracing of injected FE and CE the method of Coons and Kaplan (1950) was employed. Anti-egg-white immune serum was obtained by injecting a rabbit with 2 × 2 ml. FE and 2 × 2 ml. CE four times weekly for 6 weeks. One week after the last injection the animal was bled (serum titre 2560). A rough globulin fraction obtained by half saturation with ammonium sulphate was conjugated with fluorescein isocyanate (2 mg. isocyanate, calculated as amine, in acetone per 100 mg. protein) and dialysed and purified in the same way as the fluorescent injection material. Both were stored at − 20°. Only freeze-dried ethanol-precipitated sections were used for the antigen-antibody reactions.

Fluorescence microscopy and photomicrography.—A standard monocular microscope with a simple glass condenser was used. The light source was a 350 watt mercury vapour lamp (B.T.H. Mercra) with Wood's glass filter and a 20 mm. cuvette filled with aqueous copper sulphate. In the eye-piece 4 thicknesses of Wratten 2B gelatin filter were placed in order to cut out U-V radiation entirely.

For photography the same apparatus was used. The most suitable film was found to be Kodak Fluorodak green-sensitive. This requires a short exposure time (10–20 min.) but has unfortunately a rather large grain size so that enlargements greater than × 3 or 4 are not possible. With fine-grain films (Kodak Microfile) the exposure time was > 3 hr.

The sensitivity of Fluorodak permitted registration of the smallest amounts of yellowish-green (fluorescein) fluorescence while recording the blueish-white autofluorescence of the tissues only weakly. In the fluorescence photomicrographs which illustrate this paper the exposure times (except where specially noted) are identical; differences in intensity of the emitted light are therefore demonstrated.

RESULTS

(The results are illustrated schematically in the accompanying Diagram)

Fluorescent-labelled Protein Injection and Fluorescence Microscopy

The description of our results refers mainly to freeze-dried sections, but it was observed that the localisation of the fluorescent marker was independent of fixation, being the same in cold formalin and Helly-fixed preparations. Cold formalin fixation, however, increased the amount of bluish autofluorescence without affecting fluorescein fluorescence, while Helly fixation had a strong quenching effect on both. Comparison between Fig. 5 and 6, and between Fig. 8 and 9, should emphasise these points. Freeze-dried de-paraffinised sections are ideal for fluorescence microscopy. The primary (auto-) fluorescence of the tissue corresponds to that given by fresh unfixed frozen sections.

After injection of FEF or CEF, a suitable short time interval showed the fluorescent marker strongly concentrated by a proportion of nephrons in the renal cortex; others contained small amounts or none (Fig. 2). With increasing

time after injection a higher and higher proportion of nephrons contained the dye until after 24 hr. practically all the proximal convoluted tubules emitted a low but even greenish fluorescence.

We found strong fluorescence in the lumina of the tubules (of positive nephrons) 30 min. after injection and at the same time the brush borders became positive. Within the cell fine linear fluorescence surrounded the nuclei (see first column, 2nd row, in the Diagram) and in a photomicrograph (Fig. 3) this presents as a net-like appearance because the autofluorescence of the cytoplasm is too weak to form an image. The only other positive elements at this time are the blood vessels and the connective tissue of the renal capsule (Fig. 4). These two sites continue to be positive through the two following phases (4 and 8 hr.). After 4 hr. localisation of the marker is largely the same as in the previous phase, but the lumen contents and blood vessels become very bright. In the brush borders there is a strongly fluorescent material which is often seen in droplet form. In the photomicrographs (Fig. 5 and 6) there appear to be droplets also in the cytoplasm, but these are really situated on the surface of the lining cells.

Between 4 and 8 hr. (compare Fig. 5 and 8) no difference in localisation of the marker could be seen, but in all situations fluorescence was much brighter. For this reason, and to show clearly the strong fluorescence of brush borders and lumina, Fig. 8 was taken with a short exposure time. At this time (8 hr.) the distal tubules and Henle's loops are non-fluorescent (Fig. 16), but the contents of the collecting tubules are strongly demonstrated (Fig. 10). Their cytoplasm shows only autofluorescence.

After 18 hr. fluorescence in the injected kidney is diminishing. Nearly all the proximal convoluted tubules emit an even green light (Fig. 12) and the brush borders are distinctly less obvious. The content of the lumina is much decreased in fluorescence.

The cytoplasm of the proximal convoluted tubules continues to emit the same amount of light 30-48 hr. after injection, but the basement membranes are now strongly outlined (Fig. 14). Visible to the eye, but not shown in the photomicrograph, there now appears a fine striation in the basal parts of the cells as if the regenerating mitochondria (Fig. 15) were displacing the marker-containing cytoplasm. In the brush border zone, in the tubular lumina and in the blood vessels no fluorescence could be observed, but in the cytoplasm of the distal convoluted tubules and Henle's loops we now observed a relatively strong even fluorescence with occasional bright granules. Fig. 16 shows the distal tubules, 8 hr. after FEF, free from fluorescent marker though the blood vessels are strongly positive. Fig. 17 shows distal tubules and Henle's loops 30 hr. after FEF. The comparison is quite striking.

Three days after injection fluorescence remains qualitatively similar but quantitatively much less, while after 10 days the tubules are indistinguishable from those of controls except that there is an increase in the autofluorescence of their cytoplasm. Occasional phagocytes in the capsular connective tissue show a greenish granular fluorescence which distinguishes the injected kidneys as a whole from uninjected controls.

We observed that ribonuclease produced no change in the disposition or intensity of fluorescence in fixed tissues although some loss occurred with freeze-dried sections. Inactivated ribonuclease and incubation with water alone caused similar changes in unfixed sections. The negative effect of ribonuclease on the

fluorescent marker of fixed tissues is in sharp contrast to its effects in reversing the positive staining of the various masses and droplets with iron haematoxylin (*vide infra*).

Non-fluorescent Protein Injection. Tracing by Coons' Method

Our results with this method confirm those of Coons *et al.* (1951), but differ very greatly from those described above in animals injected with fluorescein-conjugated protein. Most impressive is the fact that the protein can only be satisfactorily demonstrated up to the 2nd hr. or, at most, in the finest traces, up to the 4th hr. The content of the tubules is an exception; it was reasonably well demonstrated up to the 8th hr. Up to 1 hr. localisation of the fluorescent antibody is mainly in the blood vessels (plasma), in the lumina of the proximal convoluted tubules and in their lining cells. In the latter the first fluorescence to appear takes the form of a ring around the nuclei, shortly afterwards (2 hr.) this has spread diffusely through the cytoplasm. The most remarkable appearance at this time and subsequently is the fluorescence of the nuclei. Fig. 7 shows the appearances of some of the tubules 2 hr. after injection of FE. Here the cytoplasm is mainly diffusely stained and unstained nuclei appear dark; ringing of the nuclei is apparent and, at the lower right and central border of the photomicrograph there are fluorescent nuclei. No such picture was seen using fluorescein-conjugated egg-white injections.

The content of the collecting tubules became weakly positive with the fluorescent antibody after 2 hr. and it was strongly positive at 4 and 8 hr., in the last instance at a time when no other constituents of the kidney gave a positive reaction.

The specificity of the reaction was controlled by using sections covered first with the unconjugated homologous antiserum (for 30 min.) and then, after washing, with the fluorescent antiserum. No secondary fluorescence was observed in these sections. As a further control, freeze-dried kidney sections from untreated animals were covered with the fluorescent antiserum. Again no secondary fluorescence was observed.

Fluorescent and Non-fluorescent Protein Injections.

Conventional Histochemical Methods

The results of applying the iron haematoxylin method to Helly-fixed sections at various intervals after protein injection are illustrated in Fig. 11, 13 and 15 in order to provide comparison with the fluorescence pictures at similar intervals. It is sufficient to state here that our results differ from those of Oliver *et al.* (1954*a*) only in our finding of significant morphological changes in the cells of the proximal convoluted tubules at an earlier stage.

After treatment of the sections with crystalline ribonuclease (6 hr., 37°) we observed that it was almost impossible to stain the mitochondria, the droplets, or the large masses of material (Fig. 11 and 18) by iron haematoxylin, Gram's method, or pyronin. A few droplets occasionally were weakly stained. The Gram-positivity of the droplets was found by Oliver *et al.* (1954*b*) to be " reduced if not entirely removed " by ribonuclease. In this respect agreement is complete; with the PAS method, however, we noted that the normal strong staining of the

brush borders of the proximal convoluted tubules was unaltered by egg-white injection while the colour of the basement membranes was increased. The droplets were usually only weakly PAS-positive with an occasional more strongly stained example. These findings are not in complete agreement with those of Oliver *et al.* (1954*b*) who suggested that the PAS reaction could easily demonstrate the difference between egg-white-induced and ovalbumin-induced droplets by the significantly higher carbohydrate content of the former.

Studies were carried out on both cold formalin-fixed and freeze-dried acetone-fixed kidneys in order to establish the relationship between the droplets and the strong intracellular non-specific esterase of the tubule cells. Using the indoxyl method of Holt and Withers (1952), we found that although esterase was present in the tubule cells containing the droplets, it was distributed diffusely throughout the cytoplasm. Other histochemical methods, particularly those for protein end groups, showed a very striking similarity between the natural droplets which occur in the rat (see below) and " unnatural " droplets produced by protein injection.

DISCUSSION

The results of the investigations presented pictorially in the second and third rows of the Diagram permit three main conclusions. First, that at all stages of protein absorption by the tubules the highest concentration of immunologically intact egg-white is found elsewhere than in the form of intracellular droplets (which in any case are not observed by the light or electron microscope much before 12 hr.). Second, that the fluorescein marker does not appear in the droplets at a stage when these can be demonstrated morphologically, and that therefore the protein moiety to which the marker remains attached is not concentrated in such a site. Third, that the immunologically intact protein is broken down extremely rapidly to fractions which no longer react with the specific antibody.

Minor conclusions are (1) that the foreign protein-induced droplets demonstrable by conventional histochemical staining methods are strikingly dependent on fixation, whereas the pattern of fluorescent protein is largely independent (Fig. 6 and 18) ; (2) that the esterase-containing predominantly protein droplets studied by Pearse (1953 ; 1954), which are a normal tubular phenomenon in older male rats and a less frequent one in older mice, are very similar histochemically to the egg-white-induced droplets except that the latter lack esterase ; (3) that the metabolism of protein takes place not only in the proximal convoluted tubules as recorded by numerous authorities, but also (Fig. 17) in the distal tubules and loops of Henle. Whether this represents absorption from the tubules or excretion into them is unknown.

We are in complete agreement with Coons in observing the deposition of egg-white traced by fluorescent antibody on and within the nuclei, an observation which is supported by the evidence of Crampton and Haurowitz (1950) that ^{131}I-labelled ovalbumin and bovine γ-globulin can be found in the nuclear fraction of homogenates of rabbit liver. We also agree with Schiller *et al.* (1952–53) that the fluorescein of the fluorescein-labelled ovalbumin fails to appear in the nuclei, although it appears in or on the nuclear membrane. An explanation suggested by these last authors is that the differential permeability of the nuclear membrane excludes fluorescein-conjugated protein. With this hypothesis we agree also.

The probable course of events in the tubular absorption of egg-white, suggested by the results of morphological, histochemical, fluorescent antibody and fluorescent marker studies, is that this protein is absorbed mainly by the brush border zone of the proximal convoluted tubules, through which it passes rapidly into the cytoplasm and thence through the nuclear membrane into the nuclear sap. The intracellular and intranuclear protein is rapidly split, so that the local concentration of immunologically definable products soon falls below the threshold at which it can be demonstrated by Coons' method. The split products remain in the cell at least up to the third day and appear to leave it by passing through the basement membrane of the tubule, and presumably through the adjacent capillary endothelium into the blood stream.

In view of the immunological work of Straus and Oliver (1955), on a purified fraction of whole kidney homogenate which contained only the droplets, the fact that in our hands these never contained either histo-immunologically demonstrable egg-white or fluorescein-marked egg-white is particularly hard to understand. These authors showed that the droplet fraction of their homogenate contained four times more egg-white than any other cellular fraction (excluding the supernatant), 6 per cent of the total protein of the droplets being reckoned as egg-white. It is unlikely that the threshold of Coons' method is above this figure. No information is available concerning the amounts of protein (antigen) demonstrable by the method, although Coons, Leduc and Connolly (1955) state that the amount of protein (antibody) demonstrable by their double technique (antibody-antigen-fluorescent antibody) is " evidently high in concentration and very small in absolute amount ".

For the fact that the fluorescent marker is not seen in the droplets there are two alternative explanations. One is that the droplets are fluorescent but that the diffuse fluorescence of the entire cytoplasm masks the slightly greater intensity of the light given off by the droplets (fluorescent light is emitted in all directions from its source). The second involves some consideration of the possible differences between unmarked and fluorescein-conjugated protein, in order to explain the lack of fluorescent material in the droplets. Although Schiller *et al.* attribute the absence of nuclear " staining " to the barrier effect of the nuclear membrane, they offer strong evidence that in other, and especially immunological, respects no differences exist between the marked and unmarked protein. We cannot exclude the operation of similar barrier effects in the membrane of the mitochondria which prevent absorption of the labelled protein or protein product.

Little is known of the rate of breakdown of protein within the renal cells, although Spector (1954) was inclined to attribute the uncombined (non-protein bound) ^{131}I of the supernatant fraction of his rat kidney homogenates to such a mechanism. It is of interest that he found that " practically no ^{131}I was demonstrable in the cell nuclei ", but this was 17 hours after the injection of protein. Spector found a tenfold increase in protein-bound ^{131}I in the mitochondrial fraction of nephrotic by comparison with normal rats. This evidence supports the immunological findings of Straus and Oliver (1955) referred to above.

On the whole, the work reported in this paper suggests that positive results, using Coons' method or fluorescein-labelled protein injections, can be regarded with confidence. Negative results, particularly if contrary to those of other acceptable techniques, should be interpreted with caution.

SUMMARY

A study of the tubular absorption of foreign protein and the intracellular development of specific droplets is reported. Young female mice were given single intra-peritoneal injections of egg-white, either unlabelled or fluorescein-labelled, and the kidneys, freeze-dried or fixed by conventional methods, were examined by three different techniques.

Two of these (tracing by fluorescent marker and tracing by fluorescent antibody) showed good agreement with each other but little agreement with the third (tracing by conventional staining or histochemical techniques for protein).

It is concluded that egg-white, passing through the glomeruli, is rapidly absorbed by the proximal convoluted tubules. It is equally rapidly broken down to smaller products and ultimately these return *viâ* the basement membrane to the blood-stream.

In the later stages of absorption the fluorescent label appears in the cytoplasm of the distal convoluted tubules. This may indicate uptake from the lumen or from the interstitial tissues.

By neither of the fluorescent methods employed could positive evidence for the presence of whole egg-white in the droplets be obtained.

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A VIRUS RELATED TO THAT CAUSING HEPATITIS IN MICE (MHV)

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APPLYING the knowledge we have gained from studies of mouse hepatitis virus (MHV) and *Eperythrozoon coccoides* (Gledhill, Dick and Andrewes, 1952; Niven, Dick, Gledhill and Andrewes, 1952; Gledhill, Dick and Niven, 1955) we have made many attempts to adapt the viruses of human hepatitis to laboratory animals. Experiments in mice in which *E. coccoides* was combined with acute-phase serum from cases of infective hepatitis (presumed virus A), or known icterogenic serum (presumed virus B) showed no evidence of any synergism between the human viruses and this parasite. Comparable experiments with *Haemobartonella muris* in rats were equally unsuccessful, nor did MHV infection of mice promote concomitant infections with the human viruses. Likewise, it proved impossible to transmit these viruses to animals which had been treated with cortisone or whose livers had been damaged with various chemical agents. In one experiment however, a viral agent was isolated from mice which had been inoculated with acute-phase serum of a case diagnosed as infective hepatitis and the properties of the agent are described in this paper. It would appear to be related to MHV; we have found no evidence that it was derived from the acute phase serum. We propose therefore to refer to the virus as MHV3 to indicate its relationship to, and to differentiate it from MHV and from the virus described by Nelson (1952) which in a subsequent communication will be referred to as MHV2 (Dick, Gledhill and Niven, to be published). To conform with this nomenclature, MHV will now be termed MHV1.

EXPERIMENTAL

Origin and Biological Properties of Virus

Ten weanling VS† mice (8–10 g. in weight, aged 20–23 days) were inoculated intraperitoneally (i.p.) with 0·1 ml. of acute phase infective hepatitis serum and 4 days later were given intraperitoneally 0·17 ml. 60 per cent glucose in distilled

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† Mice which originated from the virus-susceptible bacteria-susceptible strain of Swiss mice selectively bred by Webster (1937) were used in all experiments recorded in this paper.

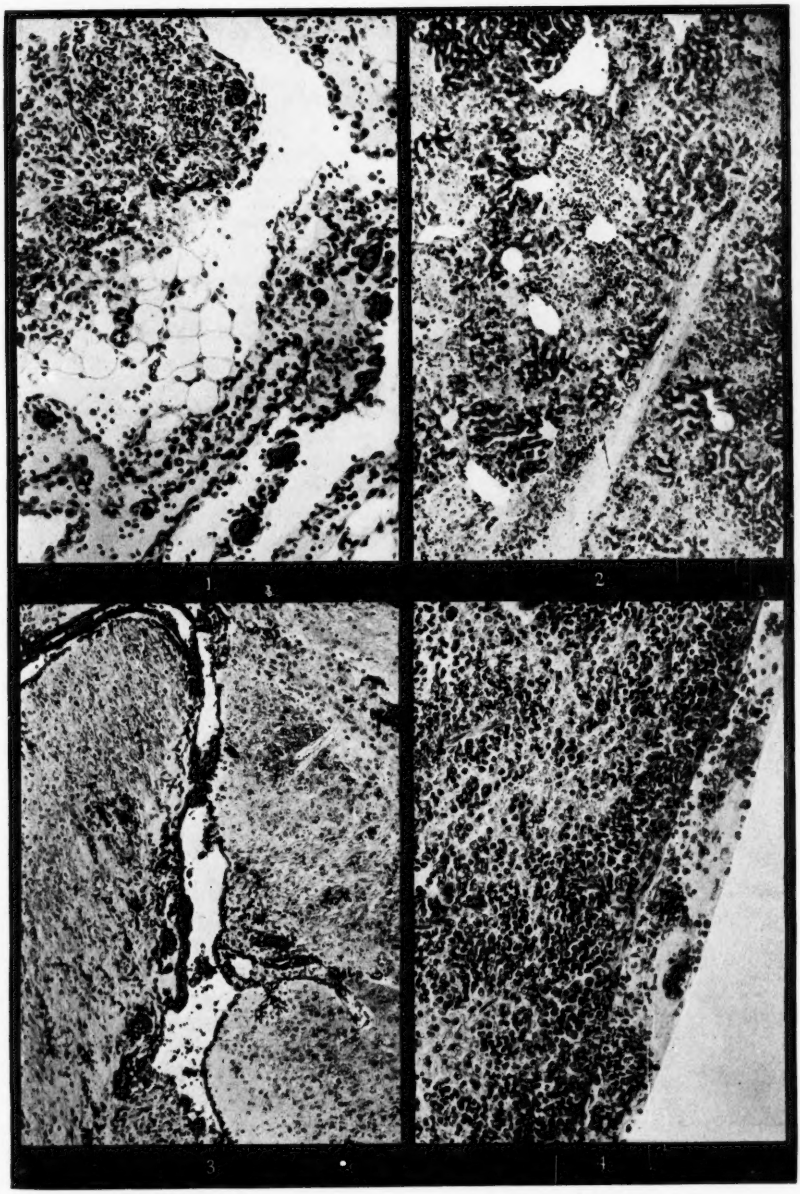
EXPLANATION OF PLATE

FIG. 1.—Omental tissue showing numerous multinucleated giant cells and areas of necrosis and haemorrhage: 48 hr. after infection. × 150.

FIG. 2.—Liver necrosis in weanling mouse 60 hr. after inoculation with MHV3. × 75.

FIG. 3.—Extensive focal periependymitis and ecnephalitis with numerous necrotic giant cells derived from ependymal epithelium. ×70.

FIG. 4.—Exuberant multinucleated giant cell reaction on the splenic surface produced by MHV1 and *E. coccoides* 3 days after previous treatment with MHV1 alone. × 200.



Dick, Niven and Gledhill.

water. Two of the mice died in shock, one was found dead with a diffuse hepatitis 4 days after the glucose injection and the remainder showed no abnormality. A 10 per cent suspension of the liver of the mouse with hepatitis was passed intraperitoneally to weanling mice (0·2 ml. per mouse) and to 2–4 day-old suckling mice (0·05 ml. per mouse). The weanling mice showed no signs of illness but when sacrificed on the 6th or 7th day 5 of 12 were found to have grossly abnormal livers. All the 2–4-day-old mice died in 5–7 days and both the original suspension and subsequent passage materials were found regularly to produce fatal hepatitis in mice of this age. The titre of 5th passage virus in suckling mice was $10^{-5.5}$ and by the 20th passage the titre was stabilised at 10^{-7}. The original virus passed in weanling mice also attained a titre of the same order ($10^{-7.5}$) by the 20th passage. Although the virus of early passage material in weanling and suckling mice failed to produce uniformly fatal hepatitis in weanlings, between the 15th and 19th passages both lines had developed a uniformly fatal action for mice of this age.

In suckling mice, extensive liver necrosis is evident within 72 hr. of inoculation and occasionally there is jaundice and a small amount of haemorrhagic exudate in the peritoneal cavity. In weanlings there may also be whitish sticky exudate on the surface of the liver and spleen and occasionally ascites. In 18–20 g. mice, which do not readily succumb to hepatitis, ascites is a more constant feature of the disease. These appearances have served to distinguish MHV3 infection in weanling mice from infections produced by a mixture of MHV1 and *E. coccoides* in mice of similar age (Gledhill *et al.*, 1955) and from the hepatitis produced by the virus described by Nelson (1952). Mice up to about 10 days of age may also be infected by subcutaneous inoculation or by feeding the virus. Infection is not transmitted by contact even to mice born in a box containing infected suckling mice. After intracerebral inoculation of infected liver suspensions, suckling and weanling mice develop a fatal hepatitis similar to that which follows i.p. inoculation.

Effect of Eperythrozöon coccoides

In view of the striking results of pre-treatment with *E. coccoides* on mouse hepatitis due to MHV1 (Gledhill *et al.*, 1955) the effect of the combination of this parasite and MHV3 was studied at early and late passage levels in both suckling and weanling mice. In *E. coccoides*-treated weanling mice, early passage MHV3 caused uniformly fatal hepatitis at all dilutions to the end-point, whereas the same virus in normal weanlings produced only a few deaths, scattered irregularly at all dilutions. In contrast, as previously stated, late passage virus, after the 20th pass, caused uniformly fatal hepatitis in the absence of *E. coccoides*. Early passage virus thus requires the presence of *E. coccoides* to produce its full pathogenic effect and resembles in this respect MHV1 of any passage level. MHV3 differs however from MHV1 in that repeated passage renders it permanently able to produce its full pathogenic action even in the absence of the mouse parasite. As might be expected, the titre of early passage virus in *E. coccoides*-inoculated mice reached approximately the same level as the late passage virus in untreated mice. Furthermore, the titre of late passage virus was not influenced by treatment of the animals with *E. coccoides*, nor is the titre of virus of any passage level in suckling mice increased by the parasite.

Neurotropic variant

About 1 in 200 infected suckling mice showed flaccid paralysis of the hind limbs, and from one such mouse of the 13th passage a neurotropic variant of the virus was obtained by intracerebral (i.c.) passage in weanling mice of unfiltered 10 per cent suspensions of brain and cord. In the first few intracerebral passes a proportion of the mice developed tremors, motor weakness or paralysis of the hind limbs, convulsions and death, 3–8 days after infection ; the livers of such mice showed severe hepatitis. By the 15th i.c. passage all mice showed signs of involvement of the central nervous system within 3–4 days, death occurring approximately 3 days later. At autopsy, some of the mice showed small areas of hepatic necrosis. The i.c. titre at this stage was $10^{-2 \cdot 7}$; by the 31st i.c. passage, the titre had increased to 10^{-4}. Hepatotropism simultaneously diminished, macroscopic focal necrosis being less constant. Intraperitoneal inoculation of the neurotropic variant produced neither signs of involvement of the central nervous system nor fatal hepatitis, although small areas of focal necrosis were usually present. Yet when the same material was inoculated into mice previously treated with *E. coccoides* fatal hepatitis invariably resulted. These results in conjunction with corresponding histological studies suggest that *E. coccoides* enables the neurotropic variant to multiply freely in liver cells, while in the absence of *E. coccoides* it would remain dormant or produce a minimal hepatic effect.

It is of interest in this connection that the neurotropic variant derived from mouse hepatitis virus (MHV1) by i.c. passage behaved in a way similar to that derived from MHV3 both in its decreasing hepatotropism following i.c. passage and in the restoration of hepatotropism in the presence of *E. coccoides*. The efforts of Gledhill and Andrewes (1951) to develop a neurotropic variant of MHV1 probably failed because *E. coccoides* in their preparations maintained the hepatotropism of that virus.

Host range

Cotton rats, white rats, guinea-pigs and rabbits showed no signs of infection after intraperitoneal inoculation of liver virus suspensions. Of all laboratory animals (other than mice) inoculated i.c. with the neurotropic variant, only cotton rats were susceptible ; 1 of 6 just weaned cotton rats died on the 9th day and another developed tremors and inco-ordination on the 12th day after inoculation. The variant was transmissible in series in cotton rats starting from brain and cord suspensions of this latter animal, but signs of infection were observed in less than half of the inoculated cotton rats.

Histopathology

Suckling mice.—The mesothelial multinucleated giant cell reaction in the abdominal and thoracic cavities occurring within 24 hr. of intraperitoneal inoculation (Niven *et al.*, 1952) is much more extensive than with MHV1 either alone or in association with *E. coccoides*, and is accompanied by abundant polymorphs, fibrinous exudate and numerous mononuclear cells. The sites of most abundant giant cell formation are the same, around the spleen and pancreas and at the base of the heart, but the process is more widespread, involving parietal, peritoneal

and pleural surfaces generally and the peritoneal surface of the stomach and duodenum. In contrast, giant cell development from venous endothelium which is so characteristic of MHV1 infection (Gledhill *et al.*, 1955) is less conspicuous except in the brain and meninges, and in veins of the spleen, pancreas, thymus and abdominal and thoracic lymphatic glands : in the hepatic, pulmonary and renal veins only an occasional giant cell of endothelial origin has been found. Areas of focal necrosis occur in omental and mesenteric tissue and on the surface of the pancreas, sometimes involving adjacent parenchymatous cells of that organ, and these foci are surrounded by a zone of intense acute inflammatory reaction, often with haemorrhage (Fig. 1). Superficial muscle fibres of the diaphragm and anterior abdominal wall may also become necrotic, and within 2 days of infection the blood-stained sticky peritoneal exudate which develops contains numerous degenerate multinucleated cells, polymorphs and mononuclear cells. Necrosis is not confined to peritoneal surfaces but appears also in lymphoid tissues, such as the Malpighian bodies of the spleen, Peyer's patches of the small intestine, the thymus, and lymphatic glands generally in the thorax and abdomen. Liver cell involvement is preceded by a remarkable development of giant cells from the covering mesothelium, especially on the under surface of the lobes and in the portal fissure, and this is quickly followed by necrosis of the surface epithelial layers. Focal necrotic lesions in the liver substance progress rapidly so that by the 3rd day following infection much of the liver has been destroyed. As in MHV1, infections cell death is preceded by the appearance of pleomorphic basophilic cytoplasmic inclusions, and as the nucleus becomes pyknotic these disappear and are replaced by intense cytoplasmic eosinophilia. Very prolific giant cell formation from the endothelium of veins in the meninges of brain and spinal cord is soon followed by the appearance of similar cells in the tissue spaces, in the Virchow-Robin space of penetrating vessels and in the ependymal lining of the ventricles : in the meninges these extra-vascular giant cells are often flattened parallel with the brain surface, and vessels in cross-section often appear completely encircled by the syncytial mass. Polymorphs, fibrinous exudate and mononuclear cells accumulate in the pia-arachnoid, the perivascular spaces within the brain substance and in the ependyma, and irregular small areas of necrosis occur later in the two latter sites.

Weanling mice.—The changes in the body cavities, organs and tissues following intraperitoneal inoculation are essentially similar to those found in suckling mice, necrosis of peritoneal and pleural surfaces, mesenteric and omental tissues and lymphoid follicles following a similar pattern. Peritoneal exudate is generally more abundant, and in the terminal stages sticky blood-stained fluid accumulates between the lobes of the liver, on the under surface of the diaphragm and between the coils of the small intestine. Liver involvement begins on the surface as a direct continuation of the mesothelial lesions and often extends quite deeply into the liver, being followed later by focal involvement throughout the entire substance (Fig. 2). Intravascular giant cell formation in the organs and tissues is limited in distribution as in suckling mice, and in the brain the process remains intravascular unaccompanied by focal meningitis or encephalitis. In animals which survive the disease, fibrous adhesions develop between the spleen, pancreas and adjacent loops of the first part of the small intestine. The capsule of the spleen and liver is thickened, and localised fibrous plaques on the surface of the spleen and on the dome of the liver have also been found.

Neurotropic variant.—The neurotropic variant described earlier produces extensive focal meningitis of the pia-arachnoid membrane of the brain and spinal cord, patchy disseminated encephalitis and focal ependymitis, the cellular reactions resembling closely those found in JHM infection of mice (Bailey, Pappenheimer, Cheever and Daniels, 1949). Polymorphs accumulate rapidly in considerable numbers in the meninges and around the penetrating blood vessels on the surface of the brain, and numerous multinucleated giant cells similar to those which develop from venous endothelium and from mesothelium appear in large numbers in the meningeal tissue spaces. The process spreads rapidly along the perivascular spaces into the brain substance, the brain now becoming studded with numerous small necrotic areas containing polymorphs and small giant cells : small haemorrhages also occur (Fig. 3). The ependymal lining of the ventricles and the choroid plexuses become extensively involved with considerable epithelial necrosis. From persisting ependymal epithelial cells, less often from those of the choroid plexuses, giant cells identical with those of endothelial cr mesothelial origin appear. In the spinal cord the general sequence of events is similar, but only occasionally have giant cells been found in the central canal. Although the anatomical distribution of the meningeal lesions varies somewhat in different animals of the same series, they tend to occur most frequently at the base of the brain, on the surface of both cerebrum and cerebellum, less often in the longitudinal fissure and in the cerebellar sulci. Within the brain substance the periependymal areas of the ventricles are always the most severely involved. Extra-vascular multinucleated giant cells occur in very large numbers ; only occasionally in the early stages of infection can small intra-vascular ones be detected. In the pleura and peritoneum, mesothelial reactions are practically absent. Giant cell formation from venous endothelium is usually found in the lungs, the veins of the spleen and pancreas and in venous channels of lymph glands. Small areas of focal necrosis occur in the liver, rarely associated with giant cell formation. It is of interest that in material taken at the 16th intracerebral passage, when hepatic lesions were often visible macroscopically, the characteristic exuberant formation of endothelial giant cells in liver and lungs, such as is found following intraperitoneal inoculation of MHV1, was observed : pleural and peritoneal reactions, however, were very inconspicuous. When combined with *E. coccoides* the cerebral lesions appear more quickly and are accompanied by extensive focal hepatitis preceded by an abundant formation of giant cells in organs and tissues except in mesothelial-lined cavities.

Interesting results follow intraperitoneal inoculation of the neurotropic variant either alone or preceded by *E. coccoides*. In the absence of *E. coccoides* an almost immediate giant cell and mono-nuclear reaction takes place in the mesothelial tissues of the splenic and pancreatic regions and of the mediastinum, but inflammatory changes and necrosis are either absent or inconspicuous ; the picture resembles that produced by MHV1 given intraperitoneally (Gledhill *et al.*, 1955). Liver lesions with giant cell development are fairly numerous but cerebral lesions are less severe, the acute inflammatory changes being practically absent, extra- and intra-vascular giant cell formation in meningeal blood vessels and in the brain substance being the most conspicuous features. When the neurotropic variant given i.p. is preceded by inoculation of *E. coccoides* liver lesions develop more rapidly and more extensively, and the details of the cellular changes in this and in other organs and tissues resemble again closely those due to MHV1, except

in the brain, where a moderate degree of acute inflammatory reaction occurs regularly in the meninges accompanied by extra- and intra-vascular giant cells: involvement of the ependyma and choroid plexuses of the ventricles has not been found.

Protective Effect of MHV3 against MHV1-E. coccoides *Mixture and MHV1 against MHV3*

Since infection with MHV1 exerts a sparing effect on the lethal action of MHV1-*E. coccoides* mixture in weanling VS mice (Gledhill *et al.*, 1952; Gledhill and Dick, 1955), experiments were carried out to determine whether MHV3 would have such an effect against the mixture and whether MHV1 would protect against the lethal action of MHV3. VS mice aged 3 weeks could not be used to test the sparing effect produced by MHV3 because the disease produced by this virus is fatal to weanling mice. Mice aged 6 and 10 weeks were therefore inoculated with 10^{-3} dilution of MHV3 and 10 days later these mice and appropriate controls which had been inoculated with normal mouse liver suspension were challenged with 10^{-2} dilution of MHV-*E. coccoides* mixture. The results of two experiments of this kind are shown in Table I. It will be seen that the mice which survived infection with MHV3 resisted the fatal action of MHV-*E. coccoides* mixture, and this might have been due to MHV3 producing resistance to the lethal action of the mixture or to MHV3 selectively eliminating the animals which would have been susceptible to the challenge inoculation. Early experiments suggested that in weanling VS mice MHV1 produced some resistance to the lethal action of MHV3. Thus, when early passage MHV3 was titrated in mice inoculated 5 days before with MHV1 and in normal mice of the same age, the total mortalities at all dilutions of MHV3 were 15/27 (56 per cent) in the pre-inoculated mice and 20/24 (83 per cent) in the normal mice. However, in experiments with late passage MHV3 which produced invariably fatal hepatitis in weanling VS mice, MHV1 inoculated 4 days and 7 days before MHV3 had no sparing effect on the fatal outcome, nor was a significant sparing effect evident

TABLE I.—*The Resistance of Mice Surviving MHV3 to the Lethal Action of MHV1 and* E. coccoides

	First inoculation.			Second inoculation.		
Expt.	Age of mice.	Agent used.	Mortality. Mice which died / Mice inoculated.	Time after first inoculation (days).	Agent used.	Mortality. Mice which died / Mice inoculated.
1	6 weeks	MHV3 10^{-3}*	22/30 (73%)	10	Mixture 10^{-2}†	0/8 (0%)
	6 „	Normal liver 10^{-3}	0/14 (0%)	10	„	10/14 (71%)
2	10 „	MHV 10^{-3}*	7/16 (44%)	9	„	0/9 (0%)
	10 „	Normal liver 10^{-3}	0/16 (0%)	9	„	8/16 (50%)

* MHV3 10^{-3} represented about 4,000 LD_{50} measured in weanling VS mice.

† Mixture means MHV1—*E. coccoides* mixture and contained more than 10,000 LD_{50} measured in weanling VS mice.

The probability of mortality differences

0/8 and 10/14 in expt. 1 occurring by chance is 0·01 ($\chi^2 = 7{\cdot}8, n = 1$).

0/9 „ 8/16 „ „ 2 „ „ „ „ 0·05 ($\chi^2 = 4{\cdot}5, n = 1$).

when such experiments were carried out with older mice (see Table II). Thus MHV1 does not produce a sparing effect upon the lethal action of MHV3.

TABLE II.—*Failure of Prior Infection with MHV1 Significantly to Lessen the Lethal Action of MHV3*

			Second inoculation.		
Expt.	Age of mice.	First inoculation agent used.	Time after first inoculation (days).	Agent used.	Mortality. Mice killed / Mice in group.	Ascites. Mice with ascites / Mice in group.
1	3 weeks	MHV1 10^{-2}*	4	MHV3 10^{-2}†	47/48 (98%)	15/48
	3 ,,	Normal liver 10^{-2}	4	MHV3 10^{-2}	48/48 (100%)	2/48
2	4 ,,	MHV1 10^{-2}*	7	MHV3 10^{-5}‡	47/52 (90%)	16/52
	4 ,,	Normal liver 10^{-2}	7	MHV3 10^{-5}	44/50 (88%)	6/50
3	7 ,,	MHV1 10^{-2}*	7	MHV3 10^{-2}†	3/20 (15%)	7/16
	7 ,,	Normal liver 10^{-2}	7	MHV3 10^{-2}	4/20 (20%)	7/16
4	7 ,,	MHV1 10^{-2}*	7	MHV3 10^{-2}†	0/25 (0%)	7/25
	7 ,,	Normal liver 10^{-2}	7	MHV3 10^{-2}	4/25 (16%)	3/21

These experiments were terminated 7 days after the second inoculation. Mice which survived for 7 days were killed and autopsied and the proportion with ascites determined.

* MHV1 10^{-2} represented about 100 LD_{50} measured in *E. coccoides*-treated weanling VS mice.

† MHV3 10^{-2} ,, ,, 40,000 LD_{50} measured in weanling VS mice.

‡ MHV3 10^{-5} ,, ,, 40 LD_{50} measured in weanling VS mice.

It will be observed from Table II that about 1/3 of the weanling mice which had been pre-treated with MHV1 showed ascites at autopsy. In contrast, a very much smaller proportion of weanling mice (8/98) which had not been infected with MHV1 before infection with MHV3 showed this condition. On the other hand, ascites was produced by MHV3 in about 30 per cent of older VS mice (10/37) and this proportion was not much increased by pre-treating them with MHV1 (14/41). In a further experiment in which mice aged 12–15 weeks were used, ascites was observed in 18 of 31 animals killed and autopsied 7 days after inoculation with MHV3, whereas it was absent in all of 15 similarly inoculated mice aged 3 weeks, all of which died of hepatitis within 6 days of infection. Passage of the ascitic fluid to weanling mice produces the typical fatal hepatitis associated with MHV3. Thus it appears that MHV3 can produce ascites as well as liver damage and in older mice, less susceptible to fatal hepatitis, ascites occurs frequently. In weanling mice treatment with MHV1 presumably causes injury to the peritoneal surface such that MHV3 is enabled to cause ascites in a much higher proportion of mice which have been pre-treated with MHV1 : in contrast, MHV1 would appear to have too slight an action on the peritoneal surface of older mice to render them more liable to ascites following infection with MHV3. From the histological aspect it is of interest that MHV1 fails to protect liver cells and mesothelial surfaces from the action of MHV3, whereas when MHV1 is followed by the lethal mixture of MHV1 and *E. coccoides* the liver cells are significantly protected and only mesothelial surfaces remain susceptible (see Fig. 4).

Physical Properties

Size.—The size of the virus was determined by filtration through gradocol membranes. The results of a typical experiment using as starting material 2μ filtrate of 10 per cent infected liver suspension of titre $10^{-4.5}$ were as follows.

Average pore diameter of filter (mμ)	190	160	130	110
Mortality ratios of inoculated mice	9/10	0/10	0/10	0/10

The size of the virus particle is estimated to be 80–120 mμ, which is the value obtained for MHV1 (Gledhill *et al.*, 1955). Filtration experiments were also made with the neurotropic variant. The cumulative results of two experiments in which lightly centrifuged mouse brain suspensions with i.c. titres of $10^{-2.5}$ and $10^{-3.2}$ were passed through gradocol filters and tested for infectivity by i.c. inoculation of mice were as follows.

Average pore diameter of filter (mμ)	300	260	230	190
Mortality ratios of inoculated mice	6/9	1/11	0/10	0/10

These results suggest a larger particle size (115–172 mμ) for the neurotropic variant. While this is quite possible (see Naudé, Madsen and Polson, 1954), the difference may be more apparent than real and may be a reflection of the lower titre of virus in the suspension of the neurotropic variant. The estimated size of the neurotropic variant of MHV1 was likewise greater than that of liver passage MHV1 (Gledhill *et al.*, 1955).

Resistance.—Ten per cent infected mouse liver suspensions in serum-saline broth and in whole serum were inactivated by heating at 56° for 30 min. Overnight exposure to a saturated solution of ether at 4° also inactivated the virus. Suspensions have retained infectivity after storage for more than 2 years at —70°.

DISCUSSION

MHV3 is similar to MHV1 in many respects, but differs primarily in its ability to produce a fatal hepatitis in weanling mice at high dilutions in the absence of *E. coccoides* and in producing ascites, particularly in older mice.

It has a more profound pathogenic action than MHV1 on the peritoneum and we have no reason to believe that the ascites is due to any agent other than MHV3. Recently, in collaboration with Dr. A. Isaacs, we have isolated from C3H mice infected with ascitic tumour S37 a hepatitis virus which has an even greater and more enduring effect on the peritoneum than MHV3 and which, like MHV3, is lethal to weanling VS mice. The hepatitis with ascites reported by Jordan and Mirick (1951) is much more chronic than the disease described here, and their causative agent does not appear to be a virus (Jordan and Mirick, 1955). We have not observed any protozoon-like structures such as have been found by Morris (1954), nor have we encountered lymphocytic choriomeningitis in any of our experiments (Traub, 1935).

The basic similarity of the lesions produced by MHV3 and MHV1 strongly supports the view that these viruses belong to the same group. This is confirmed by their corresponding heat stability and particle size and by immunological studies (Dick, Gledhill and Niven, to be published). We have never been able to repeat the recovery of MHV3 using methods identical with those which led to its isolation, nor has inoculation of mice with acute-phase sera from other cases of infective hepatitis followed by hypertonic glucose resulted in the isolation of any virus. It is not clear whether the procedure adopted induced a virulent

variant of MHV1 which is latent in the VS mouse stock, or whether MHV3 had appeared independently.

As already stated we have no evidence that MHV3 was derived from the acute phase human hepatitis serum. MHV3 differs in several respects from the causative agent of human infective hepatitis. It is more heat labile, it is inactivated by ether, and it is not neutralised by the serum of patients convalescent from infective hepatitis.

SUMMARY

In one of many unsuccessful attempts to isolate the viruses of human hepatitis in laboratory animals, a virus causing murine hepatitis was recovered from mice which had been inoculated with serum from a human case of infective hepatitis followed by hypertonic glucose solution. This virus is referred to as MHV3 and is unrelated to that of human hepatitis.

MHV3 is in many respects similar to mouse hepatitis virus (MHV, now termed MHV1) but differs in its greater virulence for weanling mice and in producing ascites in older mice and in mice pre-treated with MHV1.

Pre-treatment of mice with *E. coccoides* increased the susceptibility of weanling mice to early passage MHV3. *E. coccoides* infection had no influence on the multiplication of the virus in suckling mice, nor in weanlings inoculated with late passage virus.

A neurotropic variant of MHV3 inoculated intracerebrally in weanling mice produced fatal meningo-encephalitis with minimal liver lesions, still detectable at the 31st i.c. passage. Intraperitoneal inoculation of this variant produced mild hepatitis without signs of nervous involvement and the hepatitis was more severe in *E. coccoides*-treated mice.

The pathological effects of MHV3 and its biological and physical properties provide good evidence for the view that it is closely related to mouse hepatitis virus (MHV1).

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